II. REMARKS

A. Status of the Claims

Claims 2, 31-33, 35-51, and 60-61 were pending in the case at the time of the Office Action, with claims 5, 14-17, 23-28, and 34 having been previously withdrawn from consideration as being directed to a non-elected invention. Claims 2, 5, 14-17, 23, 25-28, 34, and 60 have been amended in the Amendment set forth herein. Claim 24 has been canceled without prejudice or disclaimer. Therefore, claims 2, 31-33, 35-51, and 60-61 are currently under consideration.

Support for the amendments to the claims can be found generally throughout the specification, such as in the claims as originally filed and page 7, line 12 – page 15, line 3. Support for "oxotechnetium" in claim 2 can be found, for example, on page 6, lines 4-7.

B. The Claim Rejections Under 35 U.S.C. §102 Are Overcome

Claims 2, 31-33, 35-38, 42-44 and 51 are rejected under 35 U.S.C. §102(b) as being anticipated by Taylor *et al.* (J. Nucl. Med., 1997, 38, p. 821-826; hereinafter "Taylor"). Applicants respectfully traverse.

Without conceding that the claims as originally written would have been anticipated by Taylor, Applicants note that claim 2, the only pending independent claim, has been amended to require that at least one of R_9 or R_{10} be aminopenciclovir, adenosine, FIAU, FIRU, IVFRU, GCV, PCV, FGCV, FPCV, FHPG, FHBG, guanine, a COX-2 inhibitor, an anti-EGF receptor, herceptin, angiostatin, thalidomide, amifostine, fullerene, lactose, leuteinizing hormone, pyridoxal, quinazoline, transferrin, or trimethyl lysine. Taylor fails to anticipate the claimed invention because Taylor does not expressly or necessarily disclose any compound that includes a moiety that is aminopenciclovir, adenosine, FIAU, FIRU, IVFRU, GCV, PCV, FGCV, FPCV,

FHPG, FHBG, guanine, a COX-2 inhibitor, an anti-EGF receptor, herceptin, angiostatin, thalidomide, amifostine, fullerene, lactose, leuteinizing hormone, pyridoxal, quinazoline, transferrin, or trimethyl lysine. See *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987) ("A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference.").

In view of the foregoing, it is respectfully requested that the rejection of claims 2, 31-33, 35-38, 42-44 and 51 under 35 U.S.C. §102(b) as being anticipated by Taylor be withdrawn.

C. The Claim Rejections Under 35 U.S.C. §103 Are Overcome

1. The Rejections Based on Iyer in View of Zareneyrizi and Further in View of Yang

Claims 2, 31-33, 35-48, 51, 60, and 61 are rejected under 35 U.S.C. §103(a) as being unpatentable over Iyer *et al.* (J. Nucl. Med., 2001, 42, p. 96-105; hereinafter "Iyer") in view of Zareneyrizi *et al.* (Anti-Cancer Drugs, 1999, 10. p. 685-692; hereinafter "Zareneyrizi") and further in view of Yang et al. (Ann. Nucl. Med. Sci., 2000, 13, p. 19-36; hereinafter "Yang"). Iyer is said to concern ^{18}F-labeled penciclovir as a probe for imaging HSV1-thymidine kinase reporter gene expression. Zareneyrizi is said to disclose synthesis of ^{99m}Tc-ethylenedicysteine (EC) colchicine to assess tumor microvasculature density. The Examiner argues that one of ordinary skill in the art would be motivated to substitute the ^{18}F label of the penciclovir of Iyer with ^{99m}Tc-EC of Zarencyrizi to lead to ^{99m} Tc-EC-penciclovir. Applicants respectfully traverse.

In rejecting claims under 35 U.S.C. §103(a), the Examiner bears the initial burden of presenting a *prima facie* case of obviousness. See *In re Rijckaert*, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993). A finding of obviousness requires that "the differences

between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains." 35 U.S.C. §103(a). In its recent decision addressing the issue of obviousness, *KSR International Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 82 U.S.P.Q.2d 1385 (2007), the Supreme Court stated that in setting forth a *prima facie* case of obviousness, it is necessary to show "some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *KSR*, 127 S.Ct. 1727 (2007) (quoting *In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006)).

The Examiner has failed to establish a *prima facie* case of obviousness because the Examiner has not set forth sufficient reason with rational underpinning as required by *KSR* to support a *prima facie* case of obviousness. Iyer teaches ^{18}F labeling of penciclovir. There is no rational basis as to why one of ordinary skill in the art would be motivated to replace the ^{18}F of Iyer with ^{99m}Tc-EC of Zareneyrizi. Nothing in Iyer teaches or suggests substituting ^{18}F with a chemical moiety, such as a radiolabeled N_2S_2 chelate, for imaging. Rather, Iyer appears to focus solely on probes with single atom radiolabels, including ^{18}I, ^{124}I, ^{3}H labeled chemical substrates. In fact, Iyer suggests that substitution with a chemical moiety would not result in an effective reporter, which actually seems to teach away from the claimed invention. For example, on page 97, second full paragraph, Iyer teaches that slight structural variations have a significant effect on biological activity. In particular, Iyer teaches that "the lack of an ether oxygen in the side chain of PCV has a significant effect on its biological properties," even though PCV is "structurally similar to GCV." Page 97, second paragraph. Thus, Iyer actually teaches away from substituting the single atom radiolabel (e.g., 18F) with a substantially larger moiety such as 99mTc-EC.

Furthermore, one of ordinary skill in the art would have understood at the time of the priority date that it would have been highly unlikely for one of ordinary skill in the art to be motivated to substitute the ^{18}F label in the penciclovir of Iyer with ^{99m}Tc-EC of Zareneyrizi because the resulting conjugate (^{99m}Tc-EC-penciclovir) would not be likely to be suitable for imaging HSV1-thymidine kinase reporter gene expression as required by Iyer since it would not be a substrate for HSV1-thymidine kinase.

Applicants herein submit the declaration of Mr. Jerry Bryant (Appendix 1; hereinafter "the Declaration"). Mr. Bryant has expertise in the synthesis and use of radionuclide-labeled imaging agents. See Declaration, ¶2-3 and Appendix A of Declaration. Mr. Bryant has declarted that "a person of ordinary skill in the art would not have been motivated to substitute the ^{18}F-labeled penciclovir probe of Iyer *et al.* with 99m-Tc-EC-aminopenciclovir because it is highly unlikely that 99m-Tc-EC-aminopenciclovir would be suitable for imaging HSV1-thymidine kinase reporter gene expression since it would not be a substrate for HSV1-thymidine kinase." Declaration, ¶5.

Mr. Bryant has noted that at the time of the invention, the prior art taught that the acyclic side chain of guanoside analogues such as penciclovir and acyclovir were known to substitute for sugar moieties. Declaration, ¶6. The side chain of Tc-EC-aminopenciclovir does not structurally resemble a sugar moiety or any part of a sugar moiety by virtue of the inclusion of the amino modification of penciclovir and by virtue of binding of EC (a molecule which does not structurally resemble a sugar moiety or part of a sugar moiety) to the amino moiety of aminopenciclovir. *Id.* He cites to information concerning the importance of structural similarity of the side chain to sugar moieties, including Elion *et al.*, Proc. Natl. Acad. Sci. USA, Vol. 74, No. 12, pp. 5716-5720, 1977 (Exhibit 1 of Declaration), Schaeffer *et al.*, J. Med. Chem. 14, 367-

369, 1971 (Exhibit 2 of Declaration), and Schaeffer *et al.*, Nature, 1978 Apr 13;272(5654):583-5 (Exhibit 3 of Declaration). Declaration, ¶6.

Furthermore, he notes that a number of acyclic guanosine analogues were known in the field at the time of the filing date of the present patent application. Declaration, ¶7. These structural analogues were known to have acyclic side chains that include at least a portion of a sugar moiety. Cited references providing examples of acyclic guanosine analogues that were known to be substrates for HSV-thymidine kinase that have side chains resembling parts of sugar moieties includeDe Clercq *et al.*, Nucleosides Nucleotides Nucleic Acids. 2001 Apr-Jul;20(4-7):271-85 (Exhibit 4 of Declaration), Ilsley *et al.*, Biochemistry. 1995 Feb 28;34(8):2504-10 (cited as Exhibit 5 in Declaration, but herein referred to as Exhibit 5a), Golbraikh *et al.*, Nucleic Acids Res. 1989 Oct 11;17(19):7965-77 (cited as Exhibit 5 in Declaration, but herein referred to as Exhibit 5b of Declaration); and Martin *et al.*, J Med Chem. 1986 Aug;29(8):1384-9 (Exhibit 6 of Declaration). Declaration, ¶7.

Thus, Mr. Bryant concludes that "[g]iven this information concerning acyclic guanosine analogues that was known in the field, a person of ordinary skill in the field of the invention would not have been motivated to employ Tc-EC-aminopenciclovir in the method of Iyer et al. to probe for HSV1-thymidine kinase expression." Declaration, ¶8.

Further, the Examiner has not cited any information to suggest that ^{99m}Tc-EC-penciclovir would be a substrate for HSV-thymidine kinase.

The Examiner argues that it was well-known in the diagnostic arts to substitute one reporter probe, or targeting moiety, for another. The Examiner has not cited any evidence to support this assertion. As set forth in Applicants' previous response, Iyer teaches that slight structural variations have a significant effect on biological activity. This teaching in Iyer

actually counters the Examiner's assertion. Further, Iyer teaches superiority of penciclovir over ganciclovir in reporting HSV-tk expression in cells, thus clearly teaching that moieties, whether for reporting or targeting, are indeed not so interchangeable.

In view of the foregoing, it is respectfully submitted that the Examiner has failed to establish a *prima facie* case of obviousness. No reasonable basis with rational underpinnings has been set forth for why one of ordinary skill in the art, in view of the teachings of these references, would combine the reference teachings to lead to the claimed invention because these references actually teach away from the claimed invention. Applicants therefore respectfully request withdrawal of the rejection under 35 U.S.C. §103(a) based on Iyer in view of Zareneyrizi and further in view of Yang.

2. The Rejections Based on Iyer in View of Zareneyrizi and Yang, and Further in View of Belinka

Claims 2, 31-33, 35-51, 60, and 61 are rejected under 35 U.S.C. §103(a) as being unpatentable over Iyer (as above) in view of Zareneyrizi (as above) and Yang (as above), and further in view of Belinka (U.S. Patent 5,609,847; hereinafter "Belinka"). The teachings of Iyer, Zareneyrizi, an Yang are as discussed above. The Examiner adds in Belinka to provide a teaching concerning kits and inclusion of gluconate or glucarate as transition chelators. Applicants respectfully traverse.

For the reasons set forth in the foregoing section, herein incorporated by reference, Iyer in view of Zareneyrizi and Yang fails to disclose the N_2S_2 chelate-targeting ligand conjugates as set forth in claim 2. Belinka fails to remedy the deficiencies of these references because it is only cited as teaching kits that include glucarate and gluconate, two transition chelators, and interchangeability of certain chelators. Without concurring with the Examiner regarding the

teachings of Belinka, Applicants note that Belinka fails to provide the missing motivation to provide for the chelator-targeting ligand conjugates as set forth in claim 2. Thus, Belinka fails to remedy the deficiencies of Iyer, Zareneyrizi, and Yang.

In view of the foregoing, it is respectfully submitted that the Examiner has failed to establish a *prima facie* case of obviousness. No reasonable basis with rational underpinnings has been set forth for why one of ordinary skill in the art, in view of the teachings of these references, would combine the reference teachings to lead to the claimed invention. Applicants therefore respectfully request withdrawal of the rejection under 35 U.S.C. §103(a) based on Iyer in view of Zareneyrizi and Yang, and further in view of Belinka.

3. The Rejections Based on Sumita in View of Yang

Claims 2, 31-33, and 38 are rejected as being unpatentable over Sumita (Radioisotopes, 1988, 37(9), p. 502-8 (abstract) in view of Yang (as per above). Applicants respectfully traverse.

Without conceding that the claims as originally written would have been obvious over Sumita in view of Yang, Applicants note that independent claim 2 has been amended to omit human serum albumin from the list of options for R_9 and R_{10}. The cited combination of references does not provide any motivation or reasonable expectation of success to provide for any of the compounds as set forth in the claims.

In view of the foregoing, it is respectfully requested that the rejection of claims 2, 31-33 and 38 under 35 U.S.C. §103(a) based on Sumita in view of Yang be withdrawn.

D. The Double Patenting Rejections Are Overcome

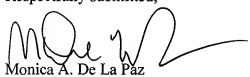
Claim 38 is provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 52-73 of copending application No. 10/672,763,

now issued as Patent No. 7,223,380. Claims 42-51 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 42-50 and 74-81 of copending application 11/405,334. Applicants understand that these rejections are only provisional. Applicants will address these rejections by filing a terminal disclaimer (filed concurrently herewith).

E. Conclusion

In view of the foregoing, it is respectfully submitted that each of the pending claims is in condition for allowance, and a Notice of Allowance is earnestly solicited. The Examiner is invited to contact the undersigned attorney at (512) 536-5639 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,



Monica A. De La Paz
Reg. No. 54,662
Attorney for Applicants

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
512.474.5201 (telephone)
512.536.4598 (fax)

Date: August 28, 2009

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Yang, *et al.*	Confirmation No. 7351
Serial No.: 10/732,919	Group Art Unit: 1618
Filed: December 10, 2003	Examiner: Schlientz, Leah H.
For: N2S2 Chelate-Targeting Ligand Conjugates	Atty. Dkt. No.: UTSC:841US

CERTIFICATE OF ELECTRONIC TRANSMISSION
37 C.F.R. § 1.8

I hereby certify that this correspondence is being electronically filed with the United States Patent and Trademark Office via EFS-Web on the date below.

Aug. 28, 2009
Date

Monica A. De La Paz

I, Jerry Bryant, do declare that:

1. I am a United States citizen residing at 6861 Staffordshire St. Houston, TX 77030.

2. I currently hold the position of Chief Technology Officer, Head of Scientific Evaluation, Division of Business Development, Cell>Point, LLC, 7120 E. Orchard Road, Suite 350, Englewood, CO 80111. A copy of my curriculum vitae, including a list of my publications, is attached as Appendix A.

3. I am skilled in the synthesis and use of radionuclide-labeled imaging agents, as evidenced by the following:

- I have worked as Chief Technology Officer of Cell>Point, LLC since 2001.
- I have been employed as the Director of Research Development of VeriMed Research Corporation in Houston, TX, from 2002-2004.

APPENDIX 1

- My duties at Cell>Point and VeriMed include participation in the development of novel radiopharmaceuticals for different diseases, such as cancer, cardiovascular disease, and diabetes. I have also been involved with studies directed to the synthesis and use of radionuclides, and studies directed to understanding the mechanism and biochemistry of the agents as it pertains to the pharmacokinetics and biodistribution of the agents in animals and humans. In addition, I have been involved in the evaluation of new technologies for the treatment of cancer, cardiovascular disease, and diabetes.
- I was employed as Chief Scientific Officer of Allcure, Inc., in Houston, TX, from May, 2001-May, 2002. My duties at Allcure included designing and marketing *in vitro* and *in vivo* services to drug and bio-tech companies for evaluating compounds of interest (nuclear medicine, SCID mouse *in vivo* service and mechanism studies).
- I have experience as a Research Assistant II in the Department of Nuclear Medicine, the Division of Diagnostic Imaging, of the University of Texas M.D. Anderson Cancer Center, from October, 2002 to June, 2003.
- I am a co-inventor of two patent applications that pertain to imaging technology, including USSN 10/703,405 ("Ethylenedicysteine (EC)-Drug Conjugates, Compositions, and Methods for Tissue Specific Disease Imaging," Yang *et al.*); and USSN 10/942,615 ("Mechanism-Based Targeted Pancreatic Beta Cell Imaging and Therapy," Yang *et al.*).
- I have been involved in funded studies pertaining to the development and evaluation of imaging agents during the past five years, including: (1) a study of CT and MRI functional agent development and evaluation supported by VeriMed Research Corporation; (2) a study of 99m-Tc-Ethylenedicysteine (EC)-Drug Conjugates for Tissue Specific Disease Imaging supported by Cell>Point, LLC; and (3) a study to compare Tc-99m-EC-deoxyglucose (EC-DG) and FDG-PET scans for the evaluation of patients

suspected of having persistent/recurrent squamous cell carcinoma of the larynx after definitive treatment with radiation therapy and the evaluation of primary lung cancer patients, sponsored by Cell>Point, LLC. See page 7 of Appendix A.

- I am a co-author of seven articles and numerous abstracts pertaining to the evaluation and testing of radiolabeled imaging agents. See pages 9-14 of Appendix A.
- I am also a co-author of two book chapters pertaining to radiolabeled imaging agents and their uses in chemistry and nuclear medicine. See page 14 of Appendix A.
- Regarding my formal education, I have a Master of Science degree (1991) in Microbiology & Cell Science and Molecular Biology from the University of Florida, and a B.S. degree (1987) in Chemistry and Biochemistry from Tennessee State University.
- I have extensive experience in cell and molecular biology, as delineated in my curriculum vitae. See pages 2-3, Appendix A.

4. I have read the above-referenced patent application and the present Office Action, and am familiar with the technology. I understand that the examiner has rejected the claims because the examiner is of the opinion that at around November, 2002 when the application was filed, a person of ordinary skill in the field of the invention would have been motivated to substitute the ^{18}F-labeled penciclovir probe of Iyer *et al.* (J. Nucl. Med., 2001, 42, p. 96-105) with 99m-Tc-ethylenedicysteine (EC)-aminopenciclovir to practice the method of Iyer *et al.* to monitor expression of HSV1-thymidine kinase. The examiner argues that the teachings of Zarenezyrizi *et al.* (Anti-Cancer Drugs, 1999, 10, p. 685-692) and Yang et al. (Ann. Nucl. Med. Sci., 2000, 13, p. 19-36) provide motivation to convert the penciclovir to amino penciclovir, and to label the aminopenciclovir with 99m-Tc-EC.

5. A person of ordinary skill in the art would not have been motivated to substitute the ^{18}F-labeled penciclovir probe of Iyer *et al.* with 99m-Tc-EC-aminopenciclovir because it is highly unlikely that 99m-Tc-EC-aminopenciclovir would be suitable for imaging HSV1-thymidine kinase reporter gene expression since it would not be a substrate for HSV1-thymidine kinase.

6. This is the case because the prior art teaches that the acyclic side chain of guanoside analogues such as penciclovir and acyclovir were known to substitute for sugar moieties. The side chain of Tc-EC-aminopenciclovir does not structurally resemble a sugar moiety or any part of a sugar moiety by virtue of the inclusion of the amino modification of penciclovir and by virtue of binding of EC (a molecule which does not structurally resemble a sugar moiety or part of a sugar moiety) to the amino moiety of aminopenciclovir. Information concerning the importance of structural similarity of the side chain to sugar moieties can be found, for example, in Elion *et al.*, Proc. Natl. Acad. Sci. USA, Vol. 74, No. 12, pp. 5716-5720, 1977 (Exhibit 1), Schaeffer *et al.*, J. Med. Chem. 14, 367-369, 1971 (Exhibit 2), and Schaeffer *et al.*, Nature, 1978 Apr 13;272(5654):583-5 (Exhibit 3).

7. Furthermore, a number of acyclic guanosine analogues were known in the field at the time of the filing date of the present patent application. These structural analogues were known to have acyclic side chains that include at least a portion of a sugar moiety. A number of references provided examples of acyclic guanosine analogues that were known to be substrates for HSV-thymidine kinase that have side chains resembling parts of sugar moieties, including De Clercq *et al.*, Nucleosides Nucleotides Nucleic Acids. 2001 Apr-Jul;20(4-7):271-85 (Exhibit 4), Ilsley *et al.*, Biochemistry. 1995 Feb 28;34(8):2504-10

(Exhibit 5), Golbraikh *et al.*, Nucleic Acids Res. 1989 Oct 11;17(19):7965-77 (Exhibit 5); and Martin *et al.*, J Med Chem. 1986 Aug;29(8):1384-9 (Exhibit 6).

8. Given this information concerning acyclic guanosine analogues that was known in the field, a person of ordinary skill in the field of the invention would not have been motivated to employ Tc-EC-aminopenciclovir in the method of Iyer et al. to probe for HSV1-thymidine kinase expression.

9. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

8/5/2009
Date

Jerry Bryant

OIPE JC
DEC 30 2004
PATENT & TRADEMARK

Revised: 12/16/04

CURRICULUM VITAE

NAME: Jerry L. Bryant, M.S.

TITLE/AFFILIATION:

(a) Primary Appointment:
Chief Technology Officer — Present
Head of Sciencitifc Evaluation
Division of Business Development
Cell>Point, LLC

(b) Joint/Adjunct Appointment:
Research Assistant II — Oct. 2002-June, 2003
Department of Experimental Nuclear Medicine
Division of Diagnostic Imaging
University of Texas M.D. Anderson Cancer Center
Houston, TX

Director of Research and Development — Present
Verimed Research Corporation
Houston, TX 77098

BIRTHDATE/PLACE: August 27, 1966, Louisiana

CITIZENSHIP: U.S. Citizen

HOME ADDRESS:
6861 Staffordshire Blvd.
Houston, Texas 77030
TELEPHONE: 713-797-6689

OFFICE ADDRESS:
Cell>Point,LLC
Division of Business Dev
7120 East Orchard Rd., Suite 350
Englewood, CO 80111
e-mail: cellpoint@swbell.net
TELEPHONE: 303-689-9693
FAX: 303-689-0198

MARITAL STATUS: Married

LICENSURES-ACTIVE: None

EDUCATION:

GRADUATE: University of Florida, M.S.
Microbiology & Cell Science w/Molecular Biology,
January, 1990-December, 1991, Gainesville, FL

UNDERGRADUATE: Tennessee State University, B.S.
Chemistry w/Biochemistry
June, 1984-May, 1987, Nashville, Tennessee

POSTGRADUATE TRAINING:
None

SPECIALITY BOARDS: None

MILITARY/GOVERNMENT: None

ACADEMIC & PROFESSIONAL APPOINTMENTS:

Chief Technology Officer
Division of Business Development
Cell>Point, LLC
(2001-present)

Research Assistant
Department of Experimental Nuclear Medicine
Division of Diagnostic Imaging
University of Texas M. D. Anderson Cancer Center,
(2002-2003)

Director of Research and Development
Division of Business Development,
Verimed Research Corporation
Houston, TX
(2002-2004)

Chief Scientific Officer
Division of Business Development
Allcure, Inc.
Houston, TX
(May, 2001-May, 2002)

President/Board Director
Allcure JEMA Medicine Services, Inc.
Medical and Research Supply Distributor
Houston, TX
(January, 1996-April, 2001)

Research Assistant II
Department of Medicine
Division of Pathology
Molecular Pathology
The University of Texas M.D. Anderson Cancer Center
Houston, TX
(November, 1994-March, 1999)

Volunteer Technician
Department of Anesthesiology
University of Florida
Gainesville, FL
(July, 1994-September, 1994)

Research Assistant
Department of Anesthesiology
University of Florida
Gainesville, FL
(August, 1992-June, 1994)

Research Associate
Department of Medicine
Division of Cardiology
Cardiovascular Disease
VA Medical Center
University of Florida
Gainesville, FL
(November, 1993-February, 1994)

Research Associate
Department of Medicine
Division of Cardiology
Cardiovascular Disease
University of Florida
Gainesville, FL
(October, 1991-March, 1992)

Research Technician
Department of Pediatric Hematology and Oncology
University of Florida
Gainesville, FL
(October, 1991-March, 1992)

Teaching Assistant
Department of Microbiology and Cell Science
University of Florida
Gainesville, FL
(January, 1991-May, 1991)

Research Assistant
Department of Microbiology and Cell Science/Molecular Biology
University of Florida
Gainesville, FL
(August, 1989-August, 1991)

Graduate Student
Department of Microbiology and Cell Science/Molecular Biology
University of Flordia
Gainesville, FL
(August, 1989-August, 1991)

Summer Miniorty Graduate Program
University of Florida
Gainesville, FL
(June, 1989-August, 1989)

a) **Consultantships**

Board of Director Member
Cell>Point, LLC
Englewood, CO
(May, 2001-Present)

Advisory Scientific Board Member
Cell>Point, LLC
Englewood, CO
(March, 2003-Present)

Board of Director Member
Verimed Research Corporation
Houston, TX
(August, 2002-Present)

ADMINISTRATIVE APPOINTMENTS AND RESPONSIBILITIES:

Director of Clinical Trial Research Protocol
Sponsor by Cell>Point, LLC
Department of Nuclear Medicine & Radiation Oncology
Division of Diagnostic Imaging
(June, 2002-present)

Director of a Pilot Biodistribution and Pharmacokinetics Study of 99mTc-EC-Annexin V in patients with Breast Cancer
Sponsor by Cell>Point, LLC
Department of Breast Medical Oncology & Division of Diagnostic Imaging
(April, 2003-May, 2004)

Director of a Pilot Imaging study of COX-2 expression with 99mTc-Celecoxib Spectro-Computer Tomography in Colorectal Cancer
Sponsor by Cell>Point, LLC
Department of Gastrointestinal Medical Oncology & Division of Diagnostic Imaging
(April, 2003-present)

COMMITTEE MEMBERSHIPS:

a) M.D. Anderson Committee Memberships/Chairmanships:

Member of the Core Logistics Committee for Clincial Trial Project
The University of Texas MD Anderson Cancer Center
(March, 2003-present)

b) Society Memberships with Offices held:

Member, American Association for Cancer Research,
(January, 2003-Present)

Member, M. D. Anderson Associates,
(October, 2002-Present)

Member, Society of Nuclear Medicine,
(June, 2002-Present)

Member, American Association for the Advancement of Science,
(August, 2001-Present)

Member, American Association of Microbiology,
(January, 1990-Present)

Member, American Chemical Society,
(June, 2002-Present)

EDITORSHIPS AND EDITORIAL BOARD MEMBERSHIPS:

Journal Reviewer:

None

HONORS AND AWARDS:

None

LECTURESHIPS AND VISITING PROFESSORSHIPS:

The 42nd American College of Cardiology Annual Scientific Session
Anaheim, CA
(March, 14-18, 1993)

ORGANIZATION OF NATIONAL OR INTERNATIONAL CONFERENCES

International:

None

PATENTS PENDING AND GRANTED:

1. Yang, D.J., Yu, D-F, Oh, C-S, and **Bryant, J.**: Ethylenedicysteine (EC)-Drug Conjugates Compositions and Methods for Tissue Specific Disease Imaging , US patent S/N 10/703,405. UTMDACC:02-073 (UTXC:758USP1), 11/7/2003 filed, US Patent (pending).

2. Yang, D.J., Oh, C-S, Kohanim, S., Yu, D-F, Azhdarinia, A. and **Bryant, J.**: Mechanism-based Targeted Pancreatic Beta Cell Imaging and Therapy, US patent S/N. UTMDACC/VeriMed Research Corporation: (10/942,615 and IPA# PCT/US04/30374), 9/10/03 filed, US patent (pending).

3. Yang, D.J., Yu, D-F, Oh, C-S, and **Bryant, J.**: N2S2 Chelate-Targeting Ligand Conjugates, US patent S/N 10/732,919. UTSC:841US-MDA02-073, 12/10/2003 filed, US patent (pending).

GRANT/CONTRACT SUPPORT: (last 5 years)

CT and MRI functional agents develpoment and evaluation (SR 2002-00007147SM). Director: **Bryant, J.L.** P.I. Yang, D.J. Supported by VeriMed Research Corporation (Houston, TX). August, 2002-August 1, 2007, $1,000,000 ($200,000/year).

^{99m}Tc-Ethylenedicysteine (EC)-Drug Conjugates for Tissue Specific Disease Imaging (LS01-212), Director: **Bryant, J.L.** P.I. Yang, D.J. Supported by Cell> Point , LLC (Englewood, CO). June 15, 2001-June 15, 2006, $1,000,000 ($200,000/year).

"Human Malignant Lymphoma Models in Immune Deficient Mice" (LS01-123), Director: **Bryant, J.L.**, P.I. Ford, RJ. Supported by VeriMed Research Corporation (Houston,TX). July, 2001-July, 2006, $1,280,000 ($256,000/year).

Assessment of Tumor Factors with 99mTc-EC-Deoxglucose for effective imaging Guided Therapy and by utilizing the EC-Technology for Targeting Specific Regulatory Functions (-01), P.I.: **Bryant, J.L.**, NIH-NCI (R21/R33) June 21, 2003-Spetember 30, 2006, $1,572,249 (Submitting).

Director **"Bryant, J.L"**., of Clinical Trial Research Protocol " Comparison of Tc-99m-EC-Deoxyglucose (EC-DG) and FDG-PET Scans for 1) the Evaluation of Patients Suspected of Having Persistent/Recurrent Squamous Cell Carcinoma of the Larynx after Definitive Treatment with Radiation Therapy and 2) the Evaluation of Primary Lung Cancer Patients", Sponsor by Cell>Point, LLC, March 10, 2003- March 10, 2005, Funding $560,000 for two years.

Director **"Bryant, J.L."** of a Pilot Biodistribution and Pharmacokinetics Study of "99mTc-EC-Annexin V in patients with Breast Cancer", Sponsor by Cell>Point, LLC, Activated May 1, 2003-May 1, 2004, Funding $25,000 for one year.

Director **"Bryant, J.L."** of a Pilot Imaging study of COX-2 expression with "99mTc-Celecoxib Spectro-Computer Tomography in Colorectal Cancer", Sponsor by Cell>Point, LLC, Activated June 1, 2003-June 1, 2004, Funding $25,000 for one year.

TEACHING EXPERIENCES:

Courses Taught:

Microbiology Laboratory, University of Florida, Nursing Students
January, 1991-May, 1991.

Training Programs: None

Other Educational Programs: None

SUPERVISORY TEACHING
Advisory Committees: None

Supervisory committees: None

Students/Postgraduates directly supervised: None

Clinical Fellows: None

Medical/Undergraduate Students: None

INVITATIONS TO NATIONAL OR INTERNATIONAL CONFERENCES: None

BIBLIOGRAPHY

PUBLISHED ARTICLES IN REFERRED JOURNALS

1. 1. **Bryant JL Jr.**, Mehta P, Von der Porten A, Mehta, JL: Co-Purification of 130 kD nitric oxide synthase and a 22 kD link protein from human neutrophils. Biochemical and Biophysical Research Communications 189: 558-564, 1992.

2. Mehta JL, **Bryant JL Jr.**, Mehta P: Reduction of Nitric oxide synthase activity in human neutrophils by oxidized low-density lipoproteins. Biochemical Pharmacology, Vol.50,No. 8,pp. 1181-1195, 1995.

3. Yang BC, **Bryant JL Jr.**, Saldeen TGP, Mehta JL: Dietary fish oil protects against ischemia-reperfusion induced myocardial dysfunction and alters vascular response without affecting nitric oxide synthesis in rats. American Heart Journal, 1993.

4. Mehta JL, Lopez LM, **Bryant JL**, Cox G: Salutary effect of antihypertensive therapy with celiprolol on nitric oxide synthase activity, superoxide anion generation and platelet aggregation in hypertensive subjects. Circulation, 1993.

5. B.C. Yang, T.G.P. Saldeen, **J.L. Bryant**, W.W. Nichols and J.L. Mehta: Long-term dietary fish oil supplementation protects against ischemia-reperfusion-induced myocardial dysfunction in isolated rat hearts. American Heart Journal, 126: 1287-92, 1993.

6. **Bryant, J.**, Pham, L., Yoshimura, L., Tamayo, A., Ordonez, N. and Ford, RJ. Development of intermediate-grade (mantle cell) and low-grade (small lymphocytic and marginal zone) human non-Hodgkin's lymphomas xenotransplanted in severe combined immunodeficiency mouse models. Laboratory Investigation, 80:557-573, 2000.

7. Yang, D.J., Kim, K-D, Schechter, N.R., Yu, D-F, Wu, P., Azhdarinia, A., Roach, J.S., Kohanim, S., Ozaki, K., Fogler, W.E., **Bryant, J.L.**, Herbst R.S., Abbruzzes J., Kim, E.E., and Podoloff, D.A. Assessment of Antiangiogenic Effect Using ^{99m}Tc-EC-Endostatin. Cancer Biotherapy and Radiopharmaceuticals. 17(2): 233-246, 2002.

8. Schechter, N.R., Yang, D.J., Azhdarinia, A., Kohanim, S., Wendt, R., Oh, C-S, Hu, M., Yu, D-F, **Bryant, J.**, Ang, K.K., Forster, K.M., Kim, E.E., and Podoloff, D.A. Assessment of EGF receptors with ^{99m}Tc-ethylenedicysteine-C225 monoclonal antibody. Anti-Cancer Drugs, 14: 49-56, 2003.

9. Yang, D.J., Kim, C-G, Schechter, N.R., Azhdarinia, A., Yu, D-F, Oh, C-S, **Bryant, J.L.**, Won, J.J., Kim, E. E., Podoloff, D.A. Imaging with ^{99m}Tc-EC-DG Targeted at the Multifunctional Glucose Transport System: Feasibility study with rodents. Radiology, 226: 465-473, 2003.

10. Yang DJ, Bryant J, Chang JY, Mendez R, Oh C-S, Yu D-F, Ito M, Azhdarinia A, Kohanim S, Kim EE, Lin E, Podoloff DA. Assessment of COX-2 expression with ^{99m}Tc-labeled celebrex. Anti-Cancer Drugs 15:255-263, 2004.

11. Yang DJ, Yukihiro M, Oh C-S, Kohanim S, Azhdarinia A, Yu D-F, Kim C-G, Ito M, Bryant JL, Kim EE, Podoloff DA. Assessment of Therapeutic Tumor Response Using ^{99m}Tc-Ethylenedicysteine-Glucosamine. Cancer Biotherapy and Radiopharm. 19(4):444-458, 2004.

12. Yang DJ, Ozaki K, Oh C-S, Azhdarinia A, Ito M, Greenwell AC, Bryant JL, Kohanim S, Kim EE. ^{99m}Tc-EC-Guanine: assessment of tuomor growth using ^{99m}Tc-EC-guanine. Chemotherapy 2004 (submitted).

13. Yang DJ, Oh C-S, Kohanim S, Ito M, Bryant JL, Yu D-F, Azhdarinia A, Greenwell AC, Kim JH, Kim EE. Imaging pancreas beta-cell sulfonylurea receptors with ^{99m}Tc-DTPA-sulfonylurea receptor agents. Diabetes 2004 (submitted).

Articles Submitted:

1. **Bryant JL Jr.**, Von der Porten A, Nicolini FA, Mehta P, Mehta JL: Oxidized low density lipoprotein decrease superoxide radical generation and increase platelet-inhibitory activity in human neutrophils.

2. Mehta P, **Bryant JL Jr.**, Mehta JL: The effect of oxidized low density lipoproteins on nitric oxide synthase in human neutrophils.

3. **Bryant JL Jr.**, Mehta P, Mehta JL: Identification of calcium-dependent nitric oxide synthase in human neutrophil membrane.

Published Abstracts:

1. **Bryant JL Jr.**, Yang BC, Mehta P, Saldeen TGP, Mehta JL: Dietary fish oil decreases superoxide radical generation without affecting nitric oxide synthase activity: a mechanism of vasorelaxation. Journal of the American College of Cardiology 21 430A, 1993.

2. Lopez LM, **Bryant JL Jr.**, Mehta JL: Effect of combined beta and alpha-adrenergic blockade on nitric oxide sythntase activity, superoxide generation and platelet aggregation in patients with hypertension. Proceeding of the American College of Clinical Pharmacology, 1993.

3. Lopez LM, **Bryant JL Jr.**, Mehta JL: Effects of therapy with celiprolol on nitric oxide synthase activity, superoxide generation and platelet aggregation in patients with hypertension. Journal of the American College of Cardiology, 1994.

4. Ford, RJ., **Bryant, J.**, Claypool, K. and Cabanillas, F: Human lymphoma models in SCID mice: Non-Hodgkin and Hodgkin's. Blood, 87, 1996.

5. Ford, RJ., Luthra R., **Bryant,J.**, Tamayo, A., Curiel,T: In vitro and in vivo models for mantle cell lymphoma. Blood, 92:314a, 1998.

6. A. Azhdarinia, D.J. Yang, S., Zakko, M., Yukihiro, D-F Yu, **J.L. Bryant**, S. Kohanim, E.E. Kim, D.A., Podoloff. Targeted tumor imaging using ^{99}mTc-EC-deoxyglucose in comparison with ^{18}F-FDG. Presented at the 49th Annual Meeting of the Society of Nuclear Medicine, Los Angeles, CA, June 15-19, 2002.

6. A. Azhdarinia, M. Yukihiro, D.J. Yang, **J.L. Bryant**, C-G Kim, D-F Yu, S. Kohanim, E.E. Kim, D.A. Podoloff. Imaging angiogenesis using ^{99}mTc-EC-endostatin. Presented at the 49th Annual Meeting of the Society of Nuclear Medicine, Los Angeles, CA, June 15-19, 2002.

7. N.R. Schechter, D.J. Yang, K. Ang, D-F Yu, L.W. Tansey, **J.L. Bryant**, E.E. Kim, D.A. Podoloff. Preliminary Tumor EGF Receptor imaging with ^{99}mTc-EC-C225. Presented at the 49th Annual Meeting of the Society of Nuclear Medicine, Los Angeles, CA, June 15-19, 2002.

8. D.J. Yang, H.A. Macapinlac, D-F. Yu, A. Azhdarinia, S. Kihanim, **J.L. Bryant**, E.E. Kim, D.A. Podoloff. Glucosamine pathway imaging using ^{99}mTc-EC-deoxyglucose in comparison with ^{18}F-FDG. Presented at the 49th Annual Meeting of the Society of Nuclear Medicine, Los Angeles, CA, June 15-19, 2002.

9. C-S Oh, D.J. Yang, C-G Kim, D-F Yu, M. Yukihiro, S. Kohanim, A. Azhdarinia, **J.L. Bryant**, E.E. Kim, D.A. Podoloff. ^{99}mTc-labeled nitroimidazole analogues for assessment of tumor hypoxia. Presented at the 49th Annual Meeting of the Society of Nuclear Medicine, Los Angeles, CA, June 15-19, 2002.

10. M. Yukihiro, D.J. Yang, T. Inoue, D-F Yu, S. Kohanim, A. Azhdarinia, **J.L. Bryant**, E.E. Kim, D.A. Podoloff. In vitro cellular uptake and in vivo biodistribution of ^{99}mTc-EC-Angiostatin. Presented at the 49th Annual Meeting of the Society of Nuclear Medicine, Los Angeles, CA, June 15-19, 2002.

11. Schechter, N. R., Yang, D. J., Ang, K., Yu, D-F, Tansey, L.W., **Bryant, J. L.**, Kim, E. E. , Podoloff, D. A. Preliminary tumor egf receptor imaging with ^{99m}Tc-EC-C225. Presented at the 49th Annual Meeting of the Society of Nuclear Medicine, Los angeles, CA June 15-19, 2002. J. Nucl. Med. 43 (5): 269, 2002 (Abstract 1088).

12. Azhdarinia, A., Yang, D. J., Zakko, S., Yukihiro, M., Yu, D-F, **Bryant, J. L.**, Kohanim, S., Kim, E. E. , Podoloff, D. A. Targeted tumor imaging using ^{99m}Tc-EC-deoxyglucose in comparison with ^{18}F-FDG. Presented at the 49th Annual Meeting of the Society of Nuclear Medicine, Los angeles, CA June 15-19, 2002. J. Nucl. Med. 43 (5): 273, 2002 (Abstract 1102).

13. Azhdarinia, A., Yukihiro, M., Yang, D. J., **Bryant, J. L.**, Kim, C-G, Yu, D-F, Kohanim, S., Kim, E. E. , Podoloff, D. A. Imaging angiogenesis using ^{99m}Tc-EC-endostatin. Presented at the 49th Annual Meeting of the Society of Nuclear Medicine, Los angeles, CA June 15-19, 2002. J. Nucl. Med. 43 (5): 121, 2002 (Abstract 435).

14. Yang, D. J., Macapinlac, H.A., Yu, D-F, Azhdarinia, A., Kohanim, S., **Bryant, J. L.**, Kim, E. E. , Podoloff, D. A. Glucosamine pathway imaging using ^{99m}Tc-EC-deoxyglucose in comparison with ^{18}F-FDG. Presented at the 49th Annual Meeting of the Society of Nuclear Medicine, Los angeles, CA June 15-19, 2002. J. Nucl. Med. 43 (5): 368, 2002 (Abstract 1478).

15. Azhdarinia, A., Yang, D. J., Schechter, N. R., Yu, D-F, **Bryant, J.**, Kohanim, S., Kim, E.E., Podoloff, D.A. ^{99m}Tc-EC-C225: An EGFR Targeting Tracer To Assess Angiogenesis. Presented at the 6th International Symposium on Technetium, Rhenium and other Metals in Chemistry and Nuclear Medicine,, Bressanone, Italy, September 4-7, 2002 (Abstract # CP5).

16. Azhdarinia, A., Yang, D. J., Yukihiro, M., Yu, D-F, **Bryant, J.**, Kim, E.E., Podoloff, D.A. ^{99m}Tc- Labeled Endostatin and Angiostatin for Angiogenesis Imaging. Presented at the 6th International Symposium on Technetium, Rhenium and other Metals in Chemistry and Nuclear Medicine, Bressanone, Italy, September 4-7, 2002 (Abstract # BP1).

17. **Jerry Bryant**, David J. Yang, Ali Azhdarinia, Edward Lin, Edmund E. Kim, Donald A. Podoloff. Radiosynthesis, Biodistribution and Planar Scintigraphy of

99mTc-Labeled Celebrex in DMBA-induced tumor bearing rats: Presented at the 94th American Association for Cancer Research 2003 Annual Meeting, Toronto, Canada, April 5-9. (Abstract # 107933).

18. M. Yukihiro, D.J. Yang, A. Azhdarinia, D-F Yu, C-G Kim, S. Kohanim, **J.L. Bryant**, E.E. Kim, D.A. Podoloff. Assessment of Tumor Growth with 99mTc-EC-Glucosamine: Presented at the 50th Annual Meeting of the Society of Nuclear Medicine, New Orleans, Louisiana, June 21-25, 2003. J. Nucl. Med. 43 (5): 368, 2002 (Abstract 1478).

19. A. Azhdarinia, D. J. Yang, M. Yukihiro, **J. L. Bryant**, D-F Yu, S. Kohanim, E. E. Kim, D. A. Podoloff. Imaging, Dosimetry and Acute Toxicity with 99mTc-EC-Deoxyglucose in Tumor-Bearing Animals. Presented at the 50th Annual Meeting of the Society of Nuclear Medicine, New Orleans, Louisiana, June 21-25, 2003. J. Nucl. Med. 43 (5): 368, 2002 (Abstract 1478).

20. A. Azhdarinia, D. J. Yang, M. Yukihiro, C. K. S. Chao, D-F Yu, **J. L. Bryant**, E. E. Kim, D. A. Podoloff. 99mTc-Labled Endostatin and Angiostatin for Angiogenesis Imaging. Presented at the 50th Annual Meeting of the Society of Nuclear Medicine, New Orleans, Louisiana, June 21-25, 2003. J. Nucl. Med. 43 (5): 368, 2002 (Abstract 1478).

21. R. Mendez, C-S Oh, A. Azhdarinia, D. J. Yang, D-F Yu, M. Yukihiro, **J. L. Bryant**, E. E. Kim, D. A. Podoloff. In-Vitro and In-Vivo evalutation of 99mTc-EC-Doxorubicin as a marker of Mdr to Doxorubicin. Presented at the 50th Annual Meeting of the Society of Nuclear Medicine, New Orleans, Louisiana, June 21-25, 2003. J. Nucl. Med. 43 (5): 368, 2002 (Abstract 1478).

22. C-S Oh, D. J. Yang, M. Yukihiro, A. Azhdarinia, D-F Yu, C-G Kim, S. Kohanim, **J. L. Bryant**, E. E. Kim, D. A. Podoloff. Synthesis and evaluation of adenosine analogue in tumor-bearing rodents for assessment of tumor cell proliferation. Presented at the 50th Annual Meeting of the Society of Nuclear Medicine, New Orleans, Louisiana, June 21-25, 2003. J. Nucl. Med. 43 (5): 368, 2002 (Abstract 1478).

23. S. Kohanim, C. Sharma, M. Yukihiro, D. J. Yang, A. Azhdarinia, D-F Yu, **J. L. Bryant**, E. E. Kim, D. A. Podoloff. Targeting Lipid metabolism with 99mTc-EC-TML: A carnitine Analogue. Presented at the 50th Annual Meeting of the Society of Nuclear Medicine, New Orleans, Louisiana, June 21-25, 2003. J. Nucl. Med. 43 (5): 368, 2002 (Abstract 1478).

24. D. J. Yang, A. Azhdarinia, M. Yukihiro, D-F Yu, C-S Oh, **J. L. Bryant**, S. Zakko, E. E. Kim, D. A. Podoloff. Assessment of tumor growth using angiogenic and apoptotic agents. Presented at the 50th Annual Meeting of the Society of Nuclear Medicine, New Orleans, Louisiana, June 21-25, 2003. J. Nucl. Med. 43 (5): 368, 2002 (Abstract 1478).

25. A. Azhdarinia, D. J. Yang, **J. L. Bryant**, M. Yukihiro, D-F Yu, C-S Oh, S. Kohanim, E. E. Kim, D. A. Podoloff. Radiosynthesis, Biodistribution and Planar Scintigraphy of

99mTc-EC-Coxi in tumor bearing animal models. Presented at the 50th Annual Meeting of the Society of Nuclear Medicine, New Orleans, Louisiana, June 21-25, 2003. J. Nucl. Med. 43 (5): 368, 2002 (Abstract 1478).

26. A. Azhdarinia, D.J. Yang, D-F Yu, C. Chao, E. Lin, J. Bryant, E.E. Kim, D.A. Podoloff. 99mTc-EC-COXi: Biodistribution and planar scintigraphy in mammary tumor-bearing animal models. Presented at the 2003 Annual Meeting of American Society of Clinical Oncology, Chicago, Illinois, May 31-June 3, 2003.

27. Yukihiro M, Yang DJ, Azhdarinia A, Yu D-F, Kim C-G, Kohanim S, Bryant JL, Kim EE, Podoloff DA. Assessment of tumor growth with ^{99m}Tc-EC-glucosamine. J Nucl Med 44 (5):299, 2003 (Abstract 1071).

28. Azhdarinia A, Yang DJ, Yukihiro M, Chao CKS, Yu D-F, Bryant JL, Kim EE, Podoloff DA. ^{99m}Tc-labled endostatin and angiostatin for angiogenesis imaging. J Nucl Med 44 (5): 302, 2003 (Abstract 1082).

29. Azhdarinia A, Yang DJ, Yukihiro M, Bryant JL, Yu D-F, Kohanim S, Kim EE, Podoloff DA. Imaging, dosimetry and acute toxicity with ^{99m}Tc-EC-deoxyglucose in tumor-bearing animals. J Nucl Med 44 (5):323, 2003 (Abstract 1158).

30. Kohanim S, Sharma C, Yukihiro M, Yang DJ, Azhdarinia A, Yu D-F, Bryant JL, Kim EE, Podoloff DA. Targeting lipid metabolism with ^{99m}Tc-EC-TML: a carnitine analogue. J Nucl Med 44 (5):301, 2003 (Abstract 1077).

31. Oh C-S, Yang DJ, Yukihiro M, Azhdarinia A, Yu D-F, Kim C-G, Kohanim S, Bryant JL, Kim EE, Podoloff DA. Synthesis and evaluation of adenosine analogue in tumor-bearing rodents for assessment of tumor cell proliferation. J Nucl Med 44 (5):301, 2003 (Abstract 1078).

32. Mendez R, Oh C-S, Azhdarinia A, Yang DJ, Yu D-F, Yukihiro M, Bryant JL, Kim EE, Podoloff DA. *In vitro* and *in vivo* evaluation of ^{99m}Tc-EC-doxorubicin as a marker of MDR to doxorubicin. J Nucl Med 44 (5): 303, 2003 (Abstract 1084).

33. Ito M, Yang DJ, Azhdarinia A, Mendez R, Kohanim S, Oh C-S, Yu D-F, Bryant JL, Chao CKS, Kim EE. PET and planar imaging of tumor hypoxia with radiolabeled metronidazole. . Proceedings of the AACR 45:104, *2004* (Abstract 946).

34. Mendez R, Bryant J, Yang DJ, Lin E, Chang JY, Ito M, Azhdarinia A, Kim EE, Podoloff DA. Imaging COX-2 expression with ^{99m}Tc-labeled celebrex. Proceedings of the AACR 45:104, *2004* (Abstract 948).

35. Rollo FD, Bryant JL, Yang DJ, Bai C, Kim EE, Yu DF, Ye J, Durbin MK, Garrard DJ, Shao L. The Complementrary Role of FDG and Tc-99m ECDG for Tumor Imaging. Proceedings of the Society of Nuclear Medicine 51st Annual Meeting, June, 2004. (Abstract No. 1062)

36. Azhdarinia A, Yang DJ, Yu DF, Oh C, Kohanim S, Mendez R, Ito M, Bryant JL, Kim EE, Podoloff DA. Local Regional Chemotherapy and Radiotherapy using an *In Situ* Hydrogel and Planar Imaging for Assessment of Tumor Growth. Proceedings of

the Society of Nuclear Medicine 51st Annual Meeting, June, 2004. (Abstract No. 1222)

37. Yang DJ, Chang JY, Mendez R, Bryant J, Azhdarina A, Oh C, Kohanim S, Ito M, Kim EE, Lin E, Podoloff DA. ^{99m}Tc-Labeled Celebrex: Synthesis, In Vitro and In Vivo Assessment of Cox-2 Expression. Proceedings of the Society of Nuclear Medicine 51st Annual Meeting, June, 2004. (Abstract No. 1406)

38. Oh C, Ozaki K, Yang DJ, Zakko S, Inoue T, Azhdarinia A, Bryant JL, Kim CG, Kohanim S, Kim EE, Podoloff DA. Assessment of Tumor Cell Proliferation with 99mTc-Labeled Adenosine and Guanine Analogues. Proceedings of the Society of Nuclear Medicine 51st Annual Meeting, June, 2004. (Abstract No. 1407)

39. Yang DJ, Oh C, Yu DF, Kohanim Azhdarinia A, Ito M, Mendez R, Chanda M. Bryant JL, Kim EE, Podoloff DA. Imaging Sulfonylurea Receptors on the Pancreas Beta-cell using Tc-99m Labeled Sulfonylurea Agents. Proceedings of the Society of Nuclear Medicine 51st Annual Meeting, June, 2004. (Abstract No. 1435)

40. Bai, C, Shao, J, Durbin, MK, Da Silva, AJ, Rollo FD, Garrard DJ, Forster, KM and Bryant, J. SPECT/CT Imaging with three-dimensional detector response correction and CT-based attenuation correction to improve SPECT Oncology. Proceedings of the Society of Nuclear Medicine 51st Annual Meeting, June, 2004. (Abstract No. 10..)

INVITED ARTICLES IN JOURNALS: None

BOOKS AND CHAPTERS:

BOOKS EDITED AND WRITTEN:

BOOK CHAPTERS:

1. Azhdarinia, A., Yang, D. J., Schechter, N. R., Yu, D-F, **Bryant, J.**, Kohanim, S., Kim, E.E., Podoloff, D.A. ^{99m}Tc-EC-C225: An EGFR Targeting Tracer To Assess Angiogenesis. *In*: M. Nicolini and U. Mazzi (eds), Technetium, Rhenium and other Metals in Chemistry and Nuclear Medicine, pp. 665-667, Padova, Italy, Servizi Grafici Editoriali snc, 2002.

2. Azhdarinia, A., Yang, D. J., Yukihiro, M., Yu, D-F, **Bryant, J.**, Kim, E.E., Podoloff, D.A. 99mTc- Labeled Endostatin and Angiostatin for Angiogenesis Imaging. *In*: M. Nicolini and U. Mazzi (eds), Technetium, Rhenium and other Metals in Chemistry and Nuclear Medicine, pp. 387-389, Padova, Italy, Servizi Grafici Editoriali snc, 2002.

REFERENCES

Richard J. Ford, M.D., Ph.D.
Professor of Hematopathology
UT MD Anderson Cancer Center
1515 Holcombe Blvd., Box 72
Houston, TX 77030 Tel. 713/792-3121

David J. Yang, Ph.D.
Associate Professor and Associate Chemist of Nuclear Medicine
Department of Nuclear Medicine
UT MD Anderson Cancer Center
1515 Holcombe Blvd., Box 59
Houston, TX 77030 Tel. 713/794-1053

E. Edmund Kim, M.D.
Professor of Radiology & Medicine
Division of Nuclear Medicine
UT MD Anderson Cancer Center
1515 Holcombe Blvd., Box 59
Houston, TX 77030 Tel. 713/794-1052

F.David Rollo, M.D., Ph.D
Chief Medical Officer
Philips Medical Systems
540 Alder Drive
Milpitas, CA 95035 Tel. 408/468-3634

Proc. Natl. Acad. Sci. USA
Vol. 74, No. 12, pp. 5716–5720, December 1977
Medical Sciences

Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine

(antiviral chemotherapy/virus-specified thymidine kinase/herpes simplex virus/virus-specified DNA polymerase/acycloguanosine triphosphate)

GERTRUDE B. ELION*, PHILLIP A. FURMAN*, JAMES A. FYFE*, PAULO DE MIRANDA*, LILIA BEAUCHAMP†, AND HOWARD J. SCHAEFFER†

Departments of * Experimental Therapy and † Organic Chemistry, Wellcome Research Laboratories, Burroughs Wellcome Company, Research Triangle Park, North Carolina 27709

Communicated by G. H. Hitchings, September 15, 1977

ABSTRACT A guanine derivative with an acyclic side chain, 2-hydroxyethoxymethyl, at position 9 has potent antiviral activity [dose for 50% inhibition (ED_{50}) = 0.1 μM] against herpes simplex virus type 1. This acyclic nucleoside analog, termed acycloguanosine, is converted to a monophosphate by a virus-specified pyrimidine deoxynucleoside (thymidine) kinase and is subsequently converted to acycloguanosine di- and triphosphates. In the uninfected host cell (Vero) these phosphorylations of acycloguanosine occur to a very limited extent. Acycloguanosine triphosphate inhibits herpes simplex virus DNA polymerase (DNA nucleotidyltransferase) 10–30 times more effectively than cellular (HeLa S3) DNA polymerase. These factors contribute to the drug's selectivity; inhibition of growth of the host cell requires a 3000-fold greater concentration of drug than does the inhibition of viral multiplication. There is, moreover, the strong possibility of chain termination of the viral DNA by incorporation of acycloguanosine.

The identity of the kinase that phosphorylates acycloguanosine was determined after separation of the cellular and virus-specified thymidine kinase activities by affinity chromatography, by reversal studies with thymidine, and by the lack of monophosphate formation in a temperature-sensitive, thymidine kinase-deficient mutant of the KOS strain of herpes simplex virus type 1 (tsA1).

The goal of antiviral chemotherapy is the discovery of antiviral agents that are specific for the inhibition of viral multiplication without affecting normal cell division. In general, compounds that have affected the multiplication of DNA viruses have also affected the replication of uninfected cells and have, therefore, been also useful in the treatment of neoplastic disease, e.g., cytosine arabinoside, adenine arabinoside, and trifluorothymidine. Iododeoxyuridine, an antiherpetic agent, is also incorporated into host cell DNA. The closely related 5-iodo-5′-amino-2′,5′-dideoxyuridine, on the other hand, has been reported to inhibit DNA synthesis of herpes simplex virus type 1 (HSV-1) selectively with little, if any, cytotoxicity to the host cells (1). Differential toxicity for herpes-infected cells has also been reported for 1-β-D-arabinofuranosyl thymine (2) and 5-ethyl-2′-deoxyuridine (3). The present paper reports the selectivity of action of a new class of antiviral agent that has extremely low toxicity for normal cells while having an inhibitory activity against HSV which is greater than that of any hitherto known compound. The most potent of these compounds is 9-(2-hydroxyethoxymethyl)guanine, a guanine derivative containing an acyclic side chain at the 9-position. This compound may be regarded as an analog of guanosine (Guo) or deoxyguanosine (dGuo) in which the 2 and 3 carbon atoms of the sugar moiety are missing (Fig. 1). It will be referred to as acycloguanosine (acyclo-Guo) in this report because of biochemical analogies.

The discovery that 9-β-D-arabinofuranosyl derivatives of 2,6-diaminopurine and of guanine possessed good antiviral activity against DNA viruses (4) suggested that purine derivatives with bases other than adenine might be potential antiviral compounds. The studies of Schaeffer *et al.* (5) had shown that acyclic side chains may substitute for sugar moieties in binding to enzymes, e.g., in a group of adenine derivatives that served as substrates for adenosine deaminase. In a continuation of this series of investigations, Schaeffer *et al.* synthesized the 9-hydroxyethoxymethyl derivatives of adenine, diaminopurine, and guanine (6), which showed activities against HSV-1 in the plaque-reduction assay with a dose required for 50% inhibition (ED_{50}) of 34, 12, and 0.1 μM, respectively. This antiviral activity of acyclo-Guo was more than two orders of magnitude greater than that of adenine arabinoside (ED_{50} = 12 μM) or guanine arabinoside (ED_{50} = 22 μM). Moreover, the compound was essentially nontoxic to the host Vero cells, showing an ED_{50} of 300 μM in 72-hr growth studies. This represents a therapeutic index in tissue culture of approximately 3000. A further examination of the specificity of this compound revealed that, although it was strongly inhibitory for both HSV-1 and HSV-2, it had essentially no activity against vaccinia virus or adenovirus. Thus, the specificity was evident not only between virus and cell, but likewise among various DNA viruses.

Acycloguanosine was active *in vivo* in mice with herpes encephalitis when the drug was given either subcutaneously or orally (6). A 1% ophthalmic ointment of acyclo-Guo cured an established herpetic infection in the eyes of rabbits (6). Herpetic lesions of guinea pig skin could be cured by topical application of the drug (6).

Metabolic studies in mice, rats, and dogs indicated that the compound was not catabolized to any significant extent *in vivo* but was excreted unchanged (ref. 6; P. de Miranda, T. Creagh, R. Sigley, H. Krasny, and G.B. Elion, unpublished). A study was, therefore, undertaken to determine the basis for the antiviral activity and high selectivity of this compound.

MATERIALS AND METHODS

Cells and Viruses. Vero cells and HeLa S-3 cells were grown in Eagle's minimal essential medium and Joklik's modified minimal essential medium, respectively. The media were

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: HSV, herpes simplex virus; TK⁻, thymidine kinase-deficient; acyclo-Guo, acycloguanosine [9-(2-hydroxyethoxymethyl)guanine; acyclo-GMP™, acycloguanosine monophosphate; acyclo-GDP, acycloguanosine diphosphate; acyclo-GTP, acycloguanosine triphosphate; ED_{50}, dose required for 50% inhibition.

EXHIBIT 1

supplemented with 10% fetal calf serum and antibiotics (50 units of penicillin plus 50 μg of streptomycin per ml). Stocks of HSV-1 strains H29 (kindly provided by D. J. Bauer), MacIntyre (kindly provided by J. S. Pagano), and KOS and a temperature-sensitive mutant of KOS, tsA1 (ts343) (kindly provided by Priscilla A. Schaffer) were prepared in Vero cell cultures and stored at −70°. Virus titrations were performed in Vero cell cultures as described by Collins and Bauer (7).

Preparation of Cell Extracts for Nucleotide Analyses by High-Pressure Liquid Chromatography. Vero cells were infected with HSV-1 with a multiplicity of infection of 5–10. One hour after infection, the 10 ml of medium was replaced by medium containing acyclo-Guo and the mixture was incubated at 36° for the time indicated. The medium was removed and the cells were trypsinized, washed with phosphate-buffered saline, and extracted with 9 volumes of cold 3.5 perchloric acid. An internal standard of 25 μl of 2 mM ITP was added to the extract to measure recovery. The acid-soluble extract was neutralized with KOH and centrifuged; the supernatant was concentrated to dryness under reduced pressure, redissolved in 0.75 ml of water, and analyzed with a Varian Aerograph LCS-1000 high-pressure liquid chromatograph fitted with a Whatman Partisil PXS 10/25 SAX column (0.46 × 25 cm). Elution was with a linear gradient of KH_2PO_4, 0.02–1.0 M at pH 3.5 with a flow rate of 30 ml/hr. Fractions (0.2 ml) were collected every 2 min for determination of radioactivity. The UV absorption was measured at 254 and 280 nm and recorded with a dual pen Honeywell Electronik 194 recorder.

Enzyme Purifications. *Separation of Viral from Host Cell Kinases.* The thymidine (dThd) kinases from HSV-1 (MacIntyre)-infected Vero cells were separated by a modification of the method of Lee and Cheng (8). The ammonium sulfate precipitation step was eliminated and most contaminating proteins were eluted from the dThd-agarose column with a high salt wash (0.9 M Tris-HCl, pH 6.8). A linear gradient of dThd containing 1 mM ATP and 1 mg of bovine serum albumin per ml eluted the dThd kinases in separate peaks.

Separation of Viral from Host Cell Polymerases. Cell extracts of HSV-1 (H29)-infected HeLa cells and mock-infected HeLa cells were prepared by the methods of Weisbach *et al.* (9) and Huang (10). The viral and cellular DNA polymerases (DNA nucleotidyltransferases) were assayed by passing the extracts through a DEAE-cellulose column, and the enzyme fractions from the second peak were pooled and applied to a phosphocellulose column (10). Samples from each fraction eluted from the column were assayed for DNA polymerase activity. The viral DNA polymerase was differentiated from the cellular α-DNA polymerase by their activities when different templates for polynucleotide synthesis were used or when 0.05 M ammonium sulfate was present in the reaction mixture (9).

Enzyme Assays. Levels of nucleoside phosphorylating activity were determined with 1 mM [^{14}C]dThd, [^{14}C]acyclo-Guo, and [^{14}C]dCyd by a procedure similar to that of Kessel (11) except that Tris-HCl, pH 7.5/5 mM $MgCl_2$/10 mM ATP was used. For assays with acyclo-Guo the DEAE-paper was washed once with water, twice with 70% ethanol/2 mM ammonium acetate/1 mM guanosine, and once with 95% ethanol. Column fractions that contained dThd were treated with 5 mM arsenate and 60 international units of thymidine phosphorylase before they were assayed for phosphorylation of acyclo-Guo or dCyd. Slices of polyacrylamide gel after electrophoresis at pH 7.5 or 8.9 (12, 13) were assayed in a similar manner. Relative reaction rates of nonradioactive nucleoside phosphorylation were determined by coupling phosphorylation to phosphate transfer from [^{14}C]phosphoenolpyruvate to ADP (ref. 14; J. A. Fyfe, unpublished).

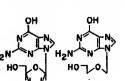


FIG. 1. Formulas for (*Left*) 2′-deoxyguanosine and (*Right*) 9-(2-hydroxyethoxymethyl)guanine.

The DNA polymerase was assayed with the reaction conditions of Weisbach *et al.* (9) and the assay procedure of Altman and Lerman (15). Briefly, a 50-μl reaction mixture containing 100 μM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM dATP, dCTP, and dGTP, 11 μg of activated calf thymus DNA, 12.5 μl of enzyme (or as stated), and 100 μM [^{3}H]-dTTP (specific activities as stated) was incubated at 37°.

Materials. The synthesis of 9-hydroxyethoxymethylguanine will be reported elsewhere. The [8-^{14}C]acyclo-Guo had a specific activity of 18 Ci/mol. The [^{3}H]acyclo-Guo was labeled in the two terminal carbons of the acyclic side chain; it had a specific activity of 51 Ci/mol. The acycloguanosine triphosphate (acyclo-GTP) used for the kinetic studies with DNA polymerase was synthesized chemically by Janet Rideout (P. A. Furman, M. St. Clair, J. Rideout, J. A. Fyfe, P. Keller, and G. B. Elion, unpublished). It was identical in all respects with the triphosphate isolated by high-pressure liquid chromatography.

RESULTS AND DISCUSSION

Anabolism of acycloguanosine in HSV-infected cells

Because of the structural resemblance of acyclo-Guo to dGuo (Fig. 1), attention was first given to enzymes that convert dGuo to its nucleotides, e.g., the deoxycytidine kinase from calf thymus, for which dGuo is a good substrate (16). Even at acyclo-Guo concentrations of 1 mM, no activity was found with this kinase (T. A. Krenitsky, unpublished). There was likewise no phosphorylation with the adenosine kinase from rabbit liver (J. Fyfe and R. Miller, unpublished). Incubation mixtures of acyclo-Guo with various cell lines were then examined. High-pressure liquid chromatography of extracts of Vero cells incubated with 1 mM acyclo-Guo, labeled either in the 8 position of the guanine ring with ^{14}C or with ^{3}H in the acyclic side chain, revealed the presence of radioactive material in the mono-, di-, and triphosphate regions of the chromatogram (Fig. 2*A*) in amounts too small for positive identification. However, the nucleotide profile of HSV-infected Vero cells treated with acyclo-Guo at a concentration of 500 μM showed a very different result (Fig. 2 *B* and *C*). Here, the amounts of mono-, di-, and triphosphates formed from the acyclic nucleoside were extremely high. The identity of the triphosphate was established in a variety of ways. The ultraviolet absorption spectrum was consistent with that of a derivative of acyclo-Guo. The specific activity of the radioactive triphosphate was the same as that of the acyclo-Guo used. In addition, peak-shift experiments showed that the radioactive triphosphate could be converted to the corresponding monophosphate derivative by the use of snake venom phosphodiesterase and to acyclo-Guo by alkaline phosphatase. It was, therefore, apparent that HSV infection had induced in these cells one or more enzymes capable of transforming acyclo-Guo into a triphosphate. Cells infected with vaccinia virus did not show this capability.

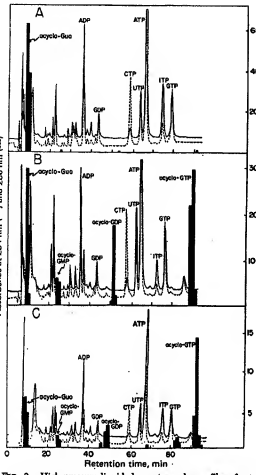


FIG. 2. High-pressure liquid chromatography profiles of extracts of uninfected and HSV-1 (H29)-infected Vero cells incubated with acyclo-Guo for 8 hr. (*A*) Uninfected cells treated with [^{3}H]acyclo-Guo (1.0 mM); (*B*) infected cells treated with [^{3}H]acyclo-Guo (0.5 mM); (*C*) infected cells treated with [8-^{14}C]acyclo-Guo (0.5 mM).

The time course of the formation of acyclo-GTP in HSV-1-infected Vero cells was investigated. Cells were infected with HSV-1 (H29) and exposed to [8-^{14}C]acyclo-Guo for various times. Acyclo-GTP formation was detectable 2 hr after infection, reached a maximum at 8 hr, and then declined but was still present in appreciable quantity at 16 hr. These findings are consistent with the fact that the enzymes involved in DNA synthesis are expressed early in the replicative cycle of HSV-1 (9, 17–19).

The extent of formation of acyclo-GTP was dependent upon the concentration of acyclo-Guo in the medium. The large difference between the uninfected and HSV-1-infected cells occurred over a wide range of drug concentration (Table 1). At 0.5 μM, which is higher than the ED_{100} for acyclo-Guo for preventing plaque formation in HSV-1-infected Vero cells, there was a 40-fold greater amount of acyclo-GTP in the infected than in uninfected cells.

Infection of Vero cells with HSV-1 resulted in a large increase in the capability of the cell to take up acyclo-Guo from the medium. The amount of uptake, as well as the degree of conversion to mono-, di-, and triphosphates, varied with the strain of HSV-1 used. Typical results for uninfected cells and cells infected with two different strains of HSV-1 and exposed to 100 μM acyclo-Guo for 7 hr are shown in Table 2. Conversion to the phosphates appears to facilitate the uptake of acyclo-Guo.

Table 1. Effect of drug concentration on the concentration of acyclo-GTP

Acyclo-Guo, μM	Acyclo-GTP, pmol/10^6 cells*	
	Vero	HSV-infected Vero
0.5	0.11	4.0
5.0	0.17	3.5
100.0	1.4	49.0
500.0	8.7	308.0

* Vero cells were infected with HSV-1 (H29), with a multiplicity of infection of 5–10. Drug was added 1 hr after infection or after uninfected cells were plated. Cells were harvested 7 hr after addition of drug.

Nucleoside phosphorylation in extracts of HSV-1-infected Vero cells

Since it was now clear that virally induced enzymes were responsible for the substantial conversion of acyclo-Guo to its phosphates, an investigation of the nature of the enzymes responsible for this conversion was undertaken. Although it has been reported (20–24) that HSV infection induces a virus-specified pyrimidine deoxynucleoside kinase, it seemed unlikely, on the basis of structural considerations, that this enzyme would be implicated. Nevertheless, cytosol fractions of uninfected and HSV-1 (H29)-infected Vero cells, prepared by the method of Cheng and Ostrander (25) but modified by the substitution of dithiothreitol for 2-mercaptoethanol and the omission of dThd, were examined for their ability to phosphorylate dThd, dCyd, and acyclo-Guo at substrate concentrations of 1 mM. The extract from uninfected cells showed a high rate of phosphorylation of dThd, a low rate for dCyd, and a barely detectable rate toward acyclo-Guo (2600, 30, and 4 pmol/min per mg of protein, respectively, at pH 7.5). However, the extracts of cells that had been infected 17 hr previously with HSV-1 (H29) showed an approximately 70-fold increase in the rate of phosphorylation of dCyd and acyclo-Guo, while maintaining a high rate of activity toward dThd (Fig. 3).‡ The activity toward dGuo in a similar extract did not increase after HSV infection.

Properties of acyclo-Guo kinase

There is strong evidence that the HSV-specified deoxypyrimidine kinase that phosphorylates both dThd and dCyd is one enzyme (25–30). It was important to determine whether this enzyme was also the one responsible for the phosphorylation of acyclo-Guo. Electrophoresis on polyacrylamide gel of the cytosol of HSV-infected Vero cells at pH 7.5 or 8.9 showed that the enzyme activities toward dThd and acyclo-Guo were in the same fractions.

Thymidine kinase from HSV-1 (MacIntyre)-infected Vero cells was purified with a thymidine-agarose affinity chromatography column. This method separated host and virus enzyme activities from other proteins and from each other. At least 95%

‡ Other experiments conducted at pH 6, the optimum pH for HSV-specified thymidine kinase (26), showed that the phosphorylation of dThd in the extracts from HSV-infected cells was due principally to HSV-specified thymidine kinase rather than to the Vero cellular enzyme. The cellular enzyme has only 11% of the activity at pH 6.0 as at pH 7.5, whereas HSV-specified enzyme is 120% as active at pH 6.0 as at pH 7.5.

Table 2. Concentration of acyclo-Guo and its derivatives in uninfected and HSV-1-infected Vero cells*

Form in extract	pmol/10^6 cells		
	Vero	HSV-1 (H29)-infected	HSV-1 (MacIntyre)-infected
Acyclo-Guo	3.4	15.1	20.1
Acyclo-GMP	2.6	14.9	4.0
Acyclo-GDP	2.0	41.5	13.4
Acyclo-GTP	1.8	71.2	90.5
Total	9.8	142.7	128.0

* Vero cells were exposed to 100 μM acyclo-Guo. Conditions were the same as in Table 1.

of the protein was eluted before a dThd gradient was introduced. Two peaks of thymidine kinase activity were eluted; the first was eluted 7 ml after the gradient was started, the second after 17–18 ml. Fractions from the first peak showed phosphorylating activities toward dThd, dCyd, and acyclo-Guo of 29, 14, and 7 pmol/hr per 20-μl sample, respectively; fractions from the second peak had activities of 2, <0.3, and <0.1, respectively. In a similar fractionation of the cytosol from uninfected Vero cells, the maximum activity with dThd as substrate occurred at 17–18 ml after the start of the gradient, with no detectable activity (level less than 2% of peak level) in fractions 6–8. The relative reaction velocities of phosphorylation of several nucleosides (at 1 mM) with the purified HSV(MacIntyre)-specified thymidine kinase were: dCyd (190), dThd (100), acyclo-Guo (36), dGuo (5), guanine arabinoside (<3), and adenine arabinoside (<3).

The evidence that acyclo-Guo was indeed being phosphorylated by the virus-specified kinase was further strengthened by several experiments showing that acyclo-GMP formation from 0.1 mM acyclo-Guo was completely prevented by 0.3 mM dThd or 8 mM dCyd, that the antiviral activity of 5 μM acyclo-Guo in Vero cells was prevented by the addition of 20 μM dThd, and that dThd interfered with the formation of acyclo-GTP in HSV-1-infected cells. In all of these studies the concentration of dThd required to produce these reversals was 3–5 times the concentration of acyclo-Guo present. By contrast, the reversal of the antiviral activity of acyclo-Guo by dGuo in the plaque-reduction assay was incomplete (25%) even at a ratio of 100:1 of dGuo:acyclo-Guo.

Finally, strong confirmatory evidence was obtained with a temperature-sensitive thymidine kinase-deficient (TK$^-$) mutant of the KOS strain of HSV-1 (tsA1), which behaves as a wild-type TK$^-$ virus at 34° (31–33). The cytosol extract of tsA1 did not phosphorylate acyclo-Guo more than the extract from uninfected cells (<10 pmol/min per mg of protein), whereas the extract from the KOS strain under the same conditions formed 1100 pmol of acyclo-GMP/min per mg of protein. In addition, extracts of Vero cells infected with the KOS strain showed a very high conversion of acyclo-Guo (100 μM) to mono-, di-, and triphosphates (80, 284, and 1236 pmol/10^6 cells, respectively, in 7 hr), whereas cells infected with tsA1 at the permissive temperature (34°) formed essentially no phosphate derivatives (total <4 pmol/10^6 cells).

Effects of acyclo-GTP on viral and cellular DNA polymerases

Although it was clear that significant quantities of acyclo-GTP were formed only in HSV-1 (TK$^+$) infected cells, it remained to be determined whether this compound was responsible for the antiviral activity of acyclo-Guo. Suggestive evidence that

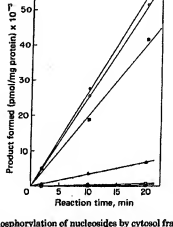


FIG. 3. Phosphorylation of nucleosides by cytosol fractions from uninfected and HSV-1 (H29)-infected Vero cells. Activities with (○,●) dThd, (□,■) dCyd, and (△,▲) acyclo-Guo were measured from uninfected (open symbols) and infected (closed symbols) cells.

acyclo-GTP was required for antiviral activity came from the findings that the tsA1 (TK$^-$) mutant of HSV-1 (KOS) described above was insensitive to acyclo-Guo at the permissive temperature. Moreover, the inability of vaccinia-infected cells to phosphorylate acyclo-Guo (J. Fyfe, unpublished) and their inability to form more acyclo-GTP than uninfected cells correlate with the lack of inhibition of the growth of vaccinia virus by 100 μM acyclo-Guo.

In order to determine the effect of acyclo-GTP on both the herpes-specified and cellular DNA polymerases, it was necessary to isolate and purify the DNA polymerase activities. The viral DNA polymerase could be differentiated from the cellular α-polymerase by the difference in their activities when different templates for polymucleotide synthesis were used or when 0.05 M ammonium sulfate was present. The cellular polymerase showed poor incorporation of [^{3}H]dTTP into acid-insoluble material with dA-dT_{12-18} as template, but high incorporation with calf thymus DNA or dC-dG_{12-18}; the activity was depressed by ammonium sulfate. The HSV-1-specified polymerase showed greatly increased activity with either dA-dT_{12-18} or dC-dG_{12-18} as template, compared with calf thymus DNA, and was stimulated by ammonium sulfate. These properties are in accordance with those previously reported for these respective polymerases (9).

The inhibitory effect of acyclo-GTP upon the isolated viral and cellular DNA polymerases was investigated by using [^{3}H]dTTP incorporation as a measure of enzyme activity. The Lineweaver–Burk plots of the data are shown in Fig. 4, with calf thymus DNA as template. From these data, which showed competitive inhibition between dGTP and acyclo-GTP, the kinetic parameters were derived. The apparent K_i (0.08 ± 0.03 μM) for acyclo-GTP for the HSV-1 (H29) polymerase was approximately $\frac{1}{30}$ that for the α-DNA polymerase of the host cell, HeLa S-3 (K_i = 2.1 ± 0.8 μM). The apparent K_m for dGTP for the viral DNA polymerase was 0.98 ± 0.13 μM, only one-third that for the cellular DNA polymerase (K_m = 1.08 ± 0.01 μM).

From the above data, it is possible to calculate whether the amount of acyclo-GTP present in HSV-1-infected cells is of the right order of magnitude to bind significantly to the viral DNA polymerase. Assuming that the concentration of acyclo-GTP

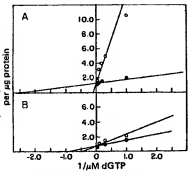


FIG. 4. Lineweaver–Burk plots showing inhibition by acyclo-GTP of (*A*) HSV-1 (H29) viral DNA polymerase and (*B*) HeLa S3 cellular DNA polymerase. ●, No acyclo-GTP; ○, 2.55 μM acyclo-GTP. The activity of the polymerase was measured by the rate of incorporation of [³H]dTTP (specific activity = 50 cpm/pmol in *A* and 36 cpm/pmol in *B*) into acid-insoluble material.

in HSV-1-infected HeLa cells is similar to that in infected Vero cells, and using a packed volume of 0.005 ml for 10^6 cells, one can calculate that a concentration of acyclo-GTP of 4 pmol/10^6 cells (found when the acyclo-Guo concentration in the medium was 0.5 μM, see Table 1) is equivalent to 0.8 μM. A concentration of 0.8 μM acyclo-GTP would be approximately 10 times greater than the apparent K_i value for the HSV-1 polymerase. On the other hand, the concentration of acyclo-GTP in uninfected cells would be 0.02 μM under the same conditions, or $\frac{1}{100}$ that of the apparent K_i for the cellular DNA polymerase.

Preliminary data indicate that acyclo-GTP is not only an inhibitor of HSV-1-specified DNA polymerase, but also a substrate for this enzyme. Incorporation of acyclo-Guo into viral DNA would be expected to be chain-terminating since no hydroxyl group corresponding to the 3′-hydroxyl group of deoxyribose is available for chain elongation. Although preliminary experiments with alkaline sucrose gradients indicate that the HSV-1 DNA is of lower molecular weight after exposure to acyclo-Guo, verification of DNA chain termination by acyclo-Guo must await the synthesis of radioactive material of higher specific activity than that presently available.

The high selectivity of acyclo-Guo for HSV-1 compared with normal host cells is now explicable on the basis of the specificity of the virus-specified thymidine kinase for acyclo-Guo and the very low activity of cellular kinases toward this compound. The specificities of the kinases that convert acyclo-GMP to acyclo-GDP and acyclo-GTP have not been examined. The virus-infected cells accumulate much more acyclo-Guo, and make approximately 40-fold more acyclo-GTP than the uninfected cells. In addition, there is a much greater sensitivity of the viral DNA polymerase compared with the cellular polymerase (30-fold in HSV-1 (H29) and HeLa cells) to acyclo-GTP. These data help to explain the 3000-fold difference between the ED_{50}s of acyclo-Guo for Vero cells and HSV-1 (H29).

We are indebted to P. Keller, M. St. Clair, and C. Lubbers for their expert technical assistance and to Dr. J. Scharver for synthesis of radioactive acyclo-Guo.

1. Cheng, Y. C., Goz, B., Neenan, J. P. Ward, D. C. & Prusoff, W. H. (1975) *J. Virol.* 15, 1284–1288.
2. Gentry, G. A. & Aswell, J. F. (1975) *Virology* 65, 294–296.
3. Cheng, Y.-C., Domin, B. A., Sharma, R. A. & Bobek, M. (1976) *Antimicrob. Agents Chemother.* 10, 119–122.
4. Elion, G. B., Rideout, J. L., de Miranda, P., Collins, P. & Bauer, D. J. (1975) *Ann. N.Y. Acad. Sci.* 255, 468–490.
5. Schaeffer, H. J., Curwara, S., Vince, R. & Bittner, S. (1971) *J. Med. Chem.* 14, 367–369.
6. Schaeffer, H. J., Beauchamp, L., de Miranda, P., Elion, G. B., Bauer, D. J. & Collins, P. (1978) *Nature*, in press.
7. Collins, P. & Bauer, D. J. (1977) *Ann. N.Y. Acad. Sci.* 284, 49–59.
8. Lee, L.-S. & Cheng, Y.-C. (1976) *J. Biol. Chem.* 251, 2600–2604.
9. Weisbach, A., Hong, S., Aucker, J. & Muller, R. (1973) *J. Biol. Chem.* 248, 6270–6277.
10. Huang, E.-S. (1975) *J. Virol.* 16, 298–310.
11. Kessel, D. (1968) *J. Biol. Chem.* 243, 4739–4744.
12. Williams, D. E. & Reisfield, R. A. (1964) *Ann. N.Y. Acad. Sci.* 121, 373–381.
13. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404–427.
14. Kornberg, A. & Pricer, W. E. (1951) *J. Biol. Chem.* 193, 481–495.
15. Altman, S. & Lerman, L. S. (1970) *J. Mol. Biol.* 50, 235–261.
16. Krenitsky, T. A., Tuttle, J. V., Koszalka, G. W., Chen, I. S., Beacham, L. M., III, Rideout, J. L. & Elion, G. B. (1976) *J. Biol. Chem.* 251, 4055–4061.
17. Cohen, G. H. (1972) *J. Virol.* 9, 408–418.
18. Keir, H. M. & Gold, E. (1963) *Biochim. Biophys. Acta* 72, 263–276.
19. Keir, H. M., Hay, J. & Morrison, J. M. (1966) *Nature* 210, 369–371.
20. Kit, S. & Dubbs, D. R. (1963) *Biochem. Biophys. Res. Commun.* 11, 55–59.
21. Dubbs, D. R. & Kit, S. (1964) *Virology* 22, 493–502.
22. Klemperer, H. C., Haynes, G. R., Shedden, W. I. H. & Watson, D. H. (1967) *Virology* 31, 120–128.
23. Buchan, A. & Watson, D. H. (1969) *J. Gen. Virol.* 4, 461–463.
24. Kit, S., Leung, W.-C., Jorgensen, G. N. & Dubbs, D. R. (1974) *Int. J. Cancer* 14, 598–610.
25. Cheng, Y.-C. & Ostrander, M. (1976) *J. Biol. Chem.* 251, 2605–2610.
26. Jamieson, A. T. & Subak-Sharpe, J. H. (1974) *J. Gen. Virol.* 24, 481–492.
27. Jamieson, A. T., Gentry, G. A. & Subak-Sharpe, J. H. (1974) *J. Gen. Virol.* 24, 465–480.
28. Leung, W.-C., Dubbs, D. R., Trkula, P. & Kit, S. (1975) *J. Virol.* 16, 486–497.
29. Thouless, M. E. & Wildy, P. (1975) *J. Gen. Virol.* 26, 159–170.
30. Cheng, Y.-C. (1976) *Biochim. Biophys. Acta* 452, 370–381.
31. Aron, G. M., Schaffer, P. A., Courtney, R. J., Benyesh-Melnick, M. & Kit, S. (1973) *Intervirology* 1, 96–109.
32. Aron, G. M., Purifoy, D. J. M. & Schaffer, P. A. (1975) *J. Virol.* 16, 498–507.
33. Schaffer, P., Vonka, V., Lewis, R. & Benyesh-Melnick, M. (1970) *Virology* 42, 1144–1146.

TABLE II
IN VITRO ANTIMICROBIAL ACTIVITY, MIN INHIB CONCN, μg/ml

Compd	SG[a]	ST	SA	SAG	BS	EC	AF	CA
1	0.1	<0.01	<0.01	0.1	1	1	10	10
3		1	100		100	10		1
5	1	1	1	1	0.1	1	100	>100
6	1	1	0.1	1	1	1	10	10
7	1	1	1	0.1	1	10	100	100
8	10	10	1	1	1	10	10	10
12		100	10		1	100		100
14		>100	100		100	>100		>100
15	>100	>100	>100	>100	>100	>100	>100	>100
16	100	100	10	100	10	100	100	>100
17	100	100	>100	100	100	>100	1	10
18	1	10	1	1	1	10	100	100

[a] SG = *Salmonella gallinarum*, ST = *S. typhimurium*, SA = *Staphylococcus aureus*, SAG = *Streptococcus agalactiae*, BS = *Bacillus subtilis*, EC = *Escherichia coli*, AF = *Aspergillus fumigatus*, CA = *Candida albicans*.

Salmonella choleraesuis infection in mice. In this test 1 and 5 were the most active of the series, but less active than 17 and 18. The α-halogenated derivatives 3, 4, 6, and 8 showed less *in vivo* antibacterial activity. Thus, the enhanced *in vitro* activity did not extend to an *in vivo* system. Replacement of a 5-nitro-2-furyl with a 5-nitro-2-thienyl group also decreased *in vivo* activity. Insufficient data are available, at present, to discuss the effect of the vinyl group on the toxicity of the series.

Surprisingly, 5 showed appreciable anticoccidial activity against *Eimeria acervulina* and *E. maxima* in chickens when administered in the feed at high levels (0.033%). The activity fell off rapidly at lower levels (0.0165, 0.0085, and 0.004%). This was particularly true with *E. acervulina* where activity was measurable only at 0.033%. Compound 5 also gave measurable protection against a mixed infection of *E. adenoeides* and *E. melagrimitis* in turkeys. This pattern of activity is in contrast to that of 18 which showed no anticoccidial activity at any level.

Experimental Section[2]

Starting Materials.—β-(5-Nitro-2-furyl)acrolein,[3] β-(5-nitro-2-furyl)-α-bromoacrolein,[3] β-(5-nitro-2-thienyl)acrolein,[4] β-(5-nitro-2-thienyl)-α-bromoacrolein,[4] and hydroxylamines,[5] not commercially available, were prepared by the methods described in the literature.

α-[β-(5-Nitro-2-heteroaryl)vinyl- or 1-bromovinyl]-*N*-substituted Nitrones. Method A.—The *N*-alkylhydroxylamine·HCl (0.01 mole) was added portionwise to a warm soln of β-(5-nitro-2-furyl)acrolein (0.01 mole) in abs EtOH (20 ml) contg $NaHCO_3$ (0.015 mole) and stirred overnight at room temperature. The mixture was worked up in the usual manner[3] and the results are shown in Table I.

Method B.—The same procedure as above was used except that 5 molar equiv of hydroxylamine alcohol oxalate was used.

Method C.—A mixture of β-(5-nitro-2-furyl)acrolein (0.005 mole) and the N-substituted hydroxylamine (0.005 mole) in C_6H_6 (20 ml) (13, 14, 15) or in a mixture (64 ml) of THF–EtOH (5:2) (16) was refluxed 0.5 hr using a Dean-Stark water separator. After cooling, the solid was filtered off and recrystd to afford the orange-red to red nitrones (Table I).

Acknowledgment.—We are indebted to W. E. Meredith, D. R. Filson, J. R. Challey, and C. A. Johnson for the biological data.

(3) Melting points were taken in open capillary tubes using a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich. Evaporation of solvents was done under reduced pressure using a rotary evaporator

Novel Substrate of Adenosine Deaminase[1a]

HOWARD J. SCHAEFFER,[1b] S. GURWARA, ROBERT VINCE, AND S. BITTNER

Department of Medicinal Chemistry, State University of New York at Buffalo, Buffalo, New York 14214

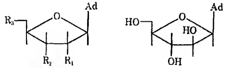
Received October 5, 1970

The structural features that determine whether a compound will be a substrate or an inhibitor of an enzyme are often difficult to untangle. For calf intestinal mucosal adenosine deaminase, it is well known that a variety of changes can be made in the carbohydrate moiety of adenosine analogs without loss of substrate activity.[2] For example, in addition to the 9-β-D-ribo configuration of adenosine (1), it is known that 9-β-D-arabino-, -xylo-, and -lyxofuranosyladenines (2, 3, 4) are all substrates of adenosine deaminase.[2] Furthermore, the 2′- and 3′-OH groups of adenosine do not play a critical role in substrate activity for it has been found that 2′-deoxy-, 3′-deoxy-, and even 2′,3′-dideoxyadenosine (5, 6, 7) undergo deamination with adenosine deaminase.[2] The 5′-OH group of adenosine and its analogs does, however, play a special role in the deamination reaction since the 5′-deoxynucleosides of adenine (8) do not undergo deamination with adenosine deaminase unless a properly positioned OH group is present at C-3′ as in 9-(5-deoxy-β-D-xylofuranosyl)adenine (9) such that the 3′-OH group can assume the function of the 5′-hydroxyl group of adenosine.[2a,d,f]

Based on these observations, we decided to synthesize an acyclic analog of adenosine containing several functional groups which appear to be important for substrate activity. The compound selected for preparation was 9-(2-hydroxyethoxymethyl)adenine (10).

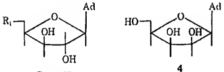
(1) (a) This work was supported by Grant T-337D from the American Cancer Society and by a Public Health Service Training Grant 5-T1-GM-00555 from the Division of Medical Sciences, Bethesda, Md. (b) Wellcome Research Laboratories, Burroughs Wellcome & Co., Research Triangle Park, North Carolina 27709.

(2) (a) R. H. Shah, H. J. Schaeffer, and D. H. Murray, *J. Pharm. Sci.*, **54**, 18 (1965). (b) J. G. Cory and R. J. Suhadolnik, *Biochemistry*, **4**, 1729 (1965). (c) A. Coddington, *Biochim. Biophys. Acta*, **99**, 442 (1965). (d) J. L. York and G. A. LePage, *Can. J. Biochem.*, **44**, 331 (1966). (e) S. Frederiksen, *Arch. Biochem. Biophys.*, **115**, 383 (1966). (f) A. Bloch, M. J. Robins, and J. R. McCarthy, *Jr., J. Med. Chem.*, **10**, 908 (1967). (g) R. M. Chassy and R. J. Suhadolnik, *J. Biol. Chem.*, **242**, 3855 (1967). (h) D. H. Murray, J. Prokop, and A. Bloch, *J. Pharm. Sci.*, **58**, 1275 (1969).



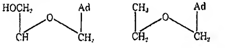
1, R_1, R_2, R_3 = OH
5, R_1 = H; R_2, R_3 = OH
6, R_2 = H; R_1, R_3 = OH
7, R_1, R_2 = H; R_3 = OH
8, R_1, R_2 = OH; R_3 = H

2



3, R_1 = OH
9, R_1 = H

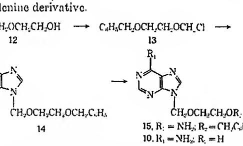
4



10 11

Furthermore, in order to assess the importance of the terminal OH of the 9 substituent of **10** on substrate activity, the deoxy derivative, 9-ethoxymethyladenine (**11**) was prepared.

The synthesis of **10**, which contains a glycosidic linkage, was initiated by converting 2-benzyloxyethanol into 1-benzyloxy-2-chloromethoxyethane. Condensation of the chloro ether **13** with 6-chloropurine followed by reaction of the condensation product with methanolic NH_3 gave the protected adenine derivative (**15**). Catalytic hydrogenolysis of the benzyl group of **15** gave the desired 9-(2-hydroxyethoxymethyl)adenine (**10**). The 6-methylamino analog was prepared by allowing the 6-chloro derivative to react with $MeNH_2$ followed by catalytic hydrogenolysis of the benzyl group to give 6-methylamino-9-(2-hydroxyethoxymethyl)purine. Finally, the synthesis of 9-ethoxymethyladenine (**11**) was accomplished by a similar procedure in which 6-chloropurine was condensed with chloromethyl ethyl ether followed by conversion with methanolic NH_3 into the adenine derivative.



Enzymatic evaluation of **10** with adenosine deaminase revealed that it was a substrate with a K_m[3] of 1.3 × 10^{-4} *M* whereas the V_{max} for **10** is only 1.4% that of adenosine. However, when the deoxy analog **11** was evaluated, it did not exhibit substrate activity and was, in fact, a relatively weak reversible inhibitor[4] of adenosine deaminase. Compound **10** can assume a conformation which is superimposable with the conformation of adenosine with respect to the adenine group, C-1′, ether oxygen, C-4′, C-5′, and the 5′-OH. Presumably, it is through this conformation that **10** complexes to the enzyme to act as a substrate of adenosine deaminase. Thus, based on the observation of the special role played by the 5′-OH for substrate activity in certain adenosine analogs, it has been possible to prepare an acyclic analog of adenosine with substrate activity for calf intestinal mucosal adenosine deaminase.

(3) For comparison, the K_m of adenosine is 3.0 × 10^{-5} *M*.
(4) The $(I/S)_{0.5}$ of **11** is 3.1.

Experimental Section[5]

9-Benzyloxyethoxyethyladenine (15).—A mixt of 2-benzyloxyethanol (9.88 g, 0.065 mole) and trioxymethylene (2.00 g, 0.022 mole) in dichloroethane (10 ml) was satd with dry HCl over a period of 2.5 hr with cooling (0°). The resulting mixt was dried ($MgSO_4$) and filtered and the solvent evapd *in vacuo*. A white oil was obtained which could not be distd *in vacuo* because of its decompn to $PhCH_2Cl$. The product (12.1 g, 93%) showed a distinctive etheric absorption in the ir. To a soln of 6-chloropurine (4.63 g, 0.03 mole) and Et_3N (3.33 g, 0.033 mole) in DMF (80 ml) was added the crude 1-benzyloxy-2-chloromethoxyethane (6.00 g, 0.03 mole). An exothermic reaction took place and Et_3NHCl immediately pptd. The reaction mixt was kept at room temp for 24 hr and filtered. The filtrate was evapd *in vacuo*, and the oily residue was chromatographed on silica gel (150 g, 100–200 mesh, column 29.5 mm id). The column was eluted with $CHCl_3$, and 55 100-ml fractions were collected. The solvent was then changed to 1% MeOH in $CHCl_3$ and another 15 100-ml fractions were collected. Eluent fractions 17–59 were combined and the solvent was evapd *in vacuo* to give an oil (14) which could not be crystd; yield, 4.2 g (44%).

A portion of the oily 6-chloro-9-benzyloxyethoxymethylpurine (250 mg, 0.78 mmole) was dissolved in 20 ml of methanolic NH_3 (20%) and heated in a stainless steel bomb at 95 ± 2° for 20 hr. On cooling, a white precipitate formed which was collected by filtration and recrystd from EtOH and then from *i*-PrOH; yield of **15**, 210 mg (90%); mp 180–181°. *Anal.* ($C_{15}H_{19}N_5O_2$) C, H, N.

9-Hydroxyethoxymethyladenine (10).—9-Benzyloxyethoxymethyladenine (400 mg, 1.34 mmoles) was dissolved in AcOH (100 ml) contg 200 mg of 5% Pd/C. The soln was hydrogenated for 20 hr, under an initial pressure of 4.2 kg/cm². The catalyst was filtered off over a Celite pad and the AcOH evaporated *in vacuo*. The remaining oil was washed several times with $CHCl_3$ and dried *in vacuo*. The crude product (150 mg, 54%) was recrystd twice from *i*-PrOH–C_6H_{14}, then from Me_2CO–C_6H_{14}, and then from Me_2CO, mp 198–199°. *Anal.* ($C_8H_{11}N_5O_2$) C, H, N.

6-Methylamino-9-(benzyloxyethoxymethyl)purine·HCl.—6-Chloro-9-(benzyloxyethoxymethyl)adenine (700 mg, 2.20 mmoles) was dissolved in EtOH (10 ml) and 15 ml of $MeNH_2$ in H_2O (40%) was added. The mixt was heated in a stainless-steel bomb for 20 hr at 95 ± 2°. The clear soln was concd *in vacuo* and the remaining material was dissolved in *i*-PrOH. Addition of dry Et_2O caused the pptn of $MeNH_2$·HCl which was filtered off. To the filtrate concd HCl (0.5 ml) was added, then the heavy ppt filtered off. The methylamino compound (0.65 g, 84%) was recrystallized from *i*-PrOH, mp 124–125°. *Anal.* ($C_{16}H_{20}ClN_5O_2$) C, H, N, Cl.

6-Methylamino-9-(2-hydroxyethoxymethyl)purine·HCl.—6-Methylamino-9-(benzyloxyethoxymethyl)purine·HCl (400 mg, 1.15 mmoles) was dissolved in glacial HOAc (100 ml) contg 200 mg of 5% Pd/C. The mixt was hydrogenated for 20 hr under an initial pressure of 4.2 kg/cm². The catalyst was removed by filtration through a Celite pad and the HOAc was evapd *in vacuo*. The remaining oil was cryst from *i*-PrOH contg 5%

(5) The melting points, unless noted otherwise, were taken in open capillary tubes on a Mel-Temp block and are uncorrected. All analytical samples had ir and uv spectra compatible with their assigned structures and moved as a single spot on tlc on Brinkman silica gel. When analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values. The analyses were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

HCl. The product (0.17 g, 60%) was recryst from i-PrOH, mp 300–304° dec. *Anal.* ($C_7H_{14}N_4O_2Cl$) C, H, N, Cl.

6-Chloro-9-(ethoxymethyl)purine.—To a suspension of 3.85 g (25.0 mmoles) of 6-chloropurine in 75 ml of DMF contg 3.0 ml (55.0 mmoles) of Et_3N was added 2.50 g (27.5 mmoles) of chloromethyl ethyl ether. After stirring the reaction mixt for 36 hr at 55–60°, it was cooled in ice and filtered. The filtrate was concd *in vacuo* to give a syrup which was chromatographed on 150.0 g of silica gel (200 mesh, column 3.0 cm i.d.) with a solvent of $CHCl_3$–MeOH (9.5:1) to give the crude product; yield, 3.12 g (56.3%), mp 72–75°. The analytical product was obtained by recrystg the crude material from Et_2O; yield, 2.26 g (40.8%); mp 79–81°. *Anal.* ($C_8H_9ClN_4O$) C, H, Cl, N.

9-Ethoxymethyladenine.—A soln of 0.64 g (3.0 mmoles) of 6-chloro-9-(ethoxymethyl)purine in 35.0 ml of 25% MeOH–NH_3 was heated at 60–70° for 18 hr in a bomb. The reaction mixt was evapd to dryness and the residue was extd with hot Me_2CO (2 × 15 ml). The Me_2CO ext was evapd *in vacuo* to give the crude product; yield 0.53 g (91.4%); mp 184–188°. One recrystn from MeOH and one from MeOH gave the pure product; yield, 0.49 g (84.5%); mp 190–192° (188° softens). *Anal.* ($C_8H_{11}N_5O$) C, H, N.

Reagents and Assay Procedures.—Adenosine deaminase (Type I, calf intestinal mucosa) was purchased from the Sigma Chemical Co. The enzyme experiments were performed at pH 7.6 in 0.05 *M* phosphate buffer at 25°. The K_m was determined by the procedure of Lineweaver and Burk.[6] The assay for the study of reversible inhibitors has previously been described[7a] and is a modification of the procedure of Kaplan[7] based on the work of Kalckar.[8]

(6) H. Lineweaver and D. Burk, *J. Amer. Chem. Soc.*, **56**, 658 (1934).
(7) N. O. Kaplan, *Methods Enzymol.*, **2**, 473 (1955).
(8) H. M. Kalckar, *J. Biol. Chem.*, **167**, 461 (1947).

Xanthoquininic Acid Derivatives

LEONARD M. RICE,* BHAGVANDAS S. SHETH, THEODORE B. ZALUCKY, AND J. W. WHEELER

College of Pharmacy and Department of Chemistry, Howard University, Washington, D. C. 20001

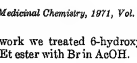
Received October 24, 1970

Some 4-aminoquinolines have a high activity in the treatment of bronchial asthma[1a–c] and malaria, as well as having antiarrhythmic[2a–c] properties. Compound I, R = CH_3, X = 2, has antiasthmatic, antihistaminic, and antiarrhythmic activity but no antimalarial activity, whereas I, X = CCCC(C), R = C_2H_5, and chloroquine have good antimalarial activity. The latter also has antiasthmatic and antiarrhythmic activity. Thus as part of an earlier study we were interested in the modification of the quinoline nucleus to produce compds of type IIc and IId.

The starting point in our synthesis was quininic acid (IIa) which was demethylated to xanthoquininic acid IIb[3] and converted into its Et ester. This phenol was then subjected to the Mannich reaction to produce the 5-substituted derivs of type IIc. The 6-dialkylaminoalkoxy derivs IId were prepared from the Na salt of the phenol with the appropriate dialkylaminoalkyl halides.

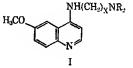
(1) (a) C. F. Geschickter, *Southern Med. J.*, **48**, 497 (1955). (b) F. Blanc, *Praxis*, **8**, 727 (1962). (c) R. C. Young, A. J. Murry, C. Carr, and K. A. Harden, *J. Nat. Med. Ass.*, **57**, 189 (1965).
(2) (a) D. M. Aviado, private communication, 1966. (b) M. A. Silver and D. M. Aviado, *Exp. Parasitol.*, **24**, 122 (1969). (c) J. W. Lawson, *J. Pharmacol. Exp. Ther.*, **160**, 22 (1968).
(3) H. John, *J. Prakt. Chem.*, **138**, 190 (1930).

During this work we treated 6-hydroxyquinoline-4-carboxylic acid Et ester with Br in AcOH. This readily



I

a, R = R′ = H; R″ = CH_3
b, R = R′ = H



II

c, R = secondary amino CH_2; R′ = H; R″ = C_2H_5
d, R = H; R′ = dialkylaminoalkyl; R″ = C_2H_5

introduced one Br atom and the product ppted out as the bromide·HBr. When boiled in H_2O this compd lost HBr and gave the free base. Introduction of the Br atom at the 5 position was confirmed by the pmr spectrum which exhibited two AB quartets in the aromatic region with $J_{2,3}$ = 4.3 Hz and $J_{7,8}$ = 9.2 Hz consistent with other quinoline derivatives.[4]

All compds prepared are listed in Table I. When evaluated for antiarrhythmic[2b–c] antimalarial,[1] and antiasthmatic[1b–c] activity none of the compds showed any appreciable activity.

Experimental Section

Elemental microanalyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. Melting points of hydrates were taken on a Fisher-Johns block, those of nonhydrates in a Thomas-Hoover capillary type apparatus. Melting points are corrected. Where analyses are indicated only by symbols of the elements or functions, analytical results obtained for those elements or functions were within ±0.4% of the theoretical values.

6-Hydroxyquinoline-4-carboxylic Acid, Xanthoquininic Acid. Quinine acid (6-methoxyquinoline-4-carboxylic acid, mp 275–278°, 101.6 g, 0.5 mole) was refluxed in 300 ml of HI, sp gr 1.7, for 12 hr. The reflux mixt was concd to 0.5 its vol, did several times its vol with H_2O, made alk with 10% NaOH, treated with Norit A, and filtered. With stirring and cooling, the filtrate was made slightly acid with AcOH. Crystn started and was completed on standing in the refrigerator overnight. The pale yellow material was filtered, resuspended in H_2O, boiled for a few min, and filtered hot. The microcryst residue (85 g, 93%) was nearly colorless, mp 339–342° dec (lit. mp 320° dec).[3] On recrystn from DMF–H_2O, or redissolving in alk and reppt'g with AcOH, a colorless product was obtained which decompd at 340–342°. If placed in the oil bath at 300° decompn points as high as 347–348° were obtained. *Anal.* Calcd for $C_{10}H_7NO_3$: C, H, N.

6-Hydroxyquinoline-4-carboxylic Acid, Et Ester.—Xanthoquininic acid (94.5 g, 0.4 mole) was placed in a 1-l. flask, and a mixt of 450 ml of abs EtOH and 50 ml of concd H_2SO_4 was added. The whole was refluxed for 8 hr, most of the EtOH was removed at the aspirator, and the viscous dark red-brown mixt was poured with stirring into 1 l. of ice H_2O. With cooling and stirring, the mixt was neutralized with 20% NaOH and placed in the refrigerator overnight. The pale yellow ppt was filtered and washed co-

(4) L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," Pergamon Press, Oxford, 1969, pp 221 and 308.
(5) T. S. Osdene, P. B. Russell, and L. Rane, *J. Med. Chem.*, **10**, 431 (1967).

articles

9-(2-Hydroxyethoxymethyl)guanine activity against viruses of the herpes group

H. J. Schaeffer, Lilia Beauchamp, P. de Miranda & Gertrude B. Elion,
Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, North Carolina

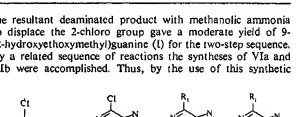
D. J. Bauer & P. Collins
Wellcome Research Laboratories, Beckenham, Kent, UK

Of a series of nucleoside analogues synthesised, 9-(2-hydroxyethoxymethyl)guanine was found to have marked antiviral activity in animal models of herpes virus infections, associated with very low toxicity.

MUCH effort has been expended in the search for effective antiviral agents, but relatively few effective agents have been found. The clinically useful compounds are 1-methylisatin 3-thiosemicarbazone (methisazone)[1], 1-aminoadamantane hydrochloride[2], 5-iodo-2'-deoxyuridine (idoxuridine)[3], 9-β-D-arabinofuranosyladenine (vidarabine)[4] and trifluorothymidine[5]. We now report on a new compound with good antiviral activity and low host toxicity—9-(2-hydroxyethoxymethyl)guanine (I), which is also referred to as acycloguanosine.

Chemistry

In recent years several interesting nucleosides have been prepared which possess antiviral activity. These nucleosides are analogues of natural products in which chemical modifications have been made in the pyrimidine or purine heterocycle, in the carbohydrate moiety, or in both[6-8]. Because earlier studies had shown that the intact cyclic carbohydrate moiety was not necessary in order to mimic nucleoside binding to enzymes[9], a programme was initiated to synthesise a range of nucleoside analogues in which the cyclic carbohydrate moiety was replaced by an acyclic side chain attached to the heterocyclic base by a glycosidic linkage. The initial synthesis of this class of compounds focused on the key intermediate 2,6-dichloro-9-(2-benzoyloxyethoxymethyl)purine (IV), which was prepared in 41% yield by the condensation of II and III in *N*,*N*-dimethylformamide in the presence of triethylamine as the acid acceptor. Because of the differences in the chemical reactivity of the 2- and 6-positions in IV, selective substitution of the 6-chloro group could be accomplished as well as simultaneous deblocking of the acyclic side chain to give V. Thus, when IV was allowed to react with methanolic ammonia at 85 °C, a good yield of V (R_1 = NH_2) was obtained. Treatment of V (R_1 = NH_2) with nitrous acid, followed by reaction of the resultant deaminated product with methanolic ammonia to displace the 2-chloro group gave a moderate yield of 9-(2-hydroxyethoxymethyl)guanine (I) for the two-step sequence. By a related sequence of reactions the syntheses of VIa and VIb were accomplished. Thus, by the use of this synthetic



VIa : R_1=NH_2, R_2=H
VIb : R_1=R_2=NH_2

procedure, a wide variety of compounds with different substituents at the 2- and 6-positions of the purine nucleus has become available.

Antiviral activity

Preliminary detection of antiviral activity was carried out *in vitro* by means of plaque inhibition tests[9,10] with monolayers of Vero cells infected with the ICI strain of type I herpes virus. Activity was measured using plaque reduction assays in the same virus-cell system, in which the test compounds were present in the agarose overlay in an appropriate series of doubling concentrations.

The plaque counts were expressed as a percentage of the number obtained in similar control cultures, and were plotted against the logarithm of the concentration of compound to give dose-response lines from which the ID_{50} values were calculated. Several standard anti-herpes compounds were assayed in a similar manner, and the ID_{50} values obtained are shown in Table 1, together with the relative potencies in comparison with idoxuridine, the compound most frequently used in clinical practice. Compound VIb is somewhat more active than vidarabine, but its activity is greatly exceeded by the guanine analogue (I), which is 160 times more active than vidarabine and 10 times more active than idoxuridine. Compound I also had a similar degree of activity (ID_{50} ~ 0.14 μM) in plaque reduction assays with type 2 strains of herpes, whereas the standard compounds generally showed lower activity. Furthermore, I has extremely low toxicity against uninfected Vero cells, which remain viable in concentrations up to 20 mM, the limit of solubility.

Preliminary studies showed that compound I inhibits the multiplication of varicella-zoster virus, cytomegalovirus and

© Macmillan Journals Ltd 1978

Table 1 Relative potencies of 9-(2-hydroxyethoxymethyl)guanine and some standard anti-herpes compounds

Compound	ID_{50} (μM)	Potency relative to idoxuridine (= 100)
Phosphonoacetic acid	57.5	1.7
VIa	40	2.5
Vidarabine	16	6
VIb	10.5	9.5
Trifluorothymidine	1.5	67
Idoxuridine	1	100
Cytarabine	0.2	500
Acycloguanosine (I)	0.1	1,000

B virus (K. R. Dumbell and E. A. Boulter, personal communications) but has no effect on vaccinia virus, adenovirus type 5 and a range of RNA viruses comprising rhinovirus 1B, Mengo, Semliki Forest, Sindbis, Bunyamwera, yellow fever, measles, respiratory syncytial virus and the NWS strain of influenza virus.

The compounds were also tested for activity *in vivo* in mice infected intracerebrally with type 1 herpes virus. Infected mouse brains were ground in a mortar and suspended in a mixture of phosphate-buffered saline and glycerol, and the stock suspensions thus obtained had LD_{50} titres of around 10^6. Groups of 10 mice were infected intracerebrally with 0.025 ml of stock suspension diluted to give doses of around 3 and 300 LD_{50}.

Test compounds were administered twice daily for 5 d by the oral route in a dose of 100 mg per kg body weight. Similar groups of infected mice were left untreated as controls. Survival times were recorded to the nearest half day over an observation period of 14 d. The results obtained in typical experiments with VIb and I are shown in Table 2. With both compounds the survival time was considerably prolonged at both dose levels of virus, and some mice in the treated groups survived the period of the experiment without showing any signs of illness. Similar results were obtained when the compounds were administered subcutaneously (s.c.).

Acycloguanosine and VIb were also evaluated against experimental herpetic keratitis in rabbits. Both corneas of a rabbit were infected with type 1 herpes virus by means of a microtrephine. The method used was a modification of that described by Jones *et al.*[11]. After 3 d, when ulceration at the sites of infection was well developed, one eye was treated and the other was left untreated as a control. Acycloguanosine was administered five times at intervals of 2 h during the working day as an ointment prepared in sterile ophthalmic petrolatum ointment base. Treatment was continued for 5 d. Each day fluorescein was instilled and drawings were made of the extent of the ulceration in both eyes, and the various signs of infection were given numerical scores according to the method of Corwin *et al.*[12]. In the untreated eye ulceration progressed until the lesions became confluent. At this stage the eye was grossly inflamed, with injection of the conjunctiva, purulent discharge,

Table 2 Treatment of herpetic encephalitis in mice with VIb and 9-(2-hydroxyethoxymethyl)guanine (I)

Compound	Virus dose (LD_{50})	Group*	Survival time (d)									
VIb	3	T	8	8	12	12	14	14	S†	S	S	S
		C	6	6	7	7	8	8	8	8	9	9
	300	T	4	4	6	6	7	7	7	7	7	8
		C	3	3	3	3	3	3	3	3	3½	3½
I	3	T	8	8	8½	10	14	14	S	S	S	S
		C	6	6	7	7	8	8	8	8	9	9
	300	T	6	6	6	7	7	7	7	8	8	8
		C	3	3	3	3	3	3	3	3	3½	3½

Data are shown for 10 individual animals per group.
*T, treated; C, control.
†S, survived with no signs of illness.

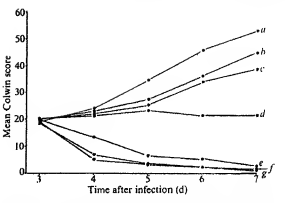


Fig. 1 Treatment of herpetic keratitis in rabbits with ophthalmic ointments containing various concentrations of acycloguanosine. *a*, Control; *b*, 0.03%; *c*, 0.1%; *d*, 0.3%; *e*, 1.0%; *f*, 2.0%; *g*, 3.0% acycloguanosine. Treatment was begun on day 3.

opacity of the cornea and extensive ulceration. In the treated eye the extent of the ulceration regressed after the first day of treatment, and by the 5th day healing was complete, apart from some residual injection of the conjunctiva. The mean Corwin scores recorded in experiments with ointments of different concentrations of acycloguanosine carried out on a total of 60 rabbits are given in Fig. 1, which shows that this compound produces a satisfactory cure of herpetic keratitis in concentrations of 1% or higher. Similar results were obtained with aqueous solutions of VIb in concentrations of 1% and higher.

The effect of the compounds on cutaneous herpes infections was studied in guinea pigs. Both flanks of the animal were shaved and further epilated with a barium sulphide preparation. The skin was then washed and dried, and 4 h later a suspension of type 1 herpes virus containing 10^8 plaque-forming units (PFU) per ml was inoculated at eight sites on both flanks by scarification. After 18 h, when lesions had begun to develop, those on one flank were treated twice daily with a water-soluble ointment formulation of acycloguanosine, and the lesions on the other flank were left untreated as a control. Treatment was continued for 3 d and the lesions on both flanks were photographed. The results of treatment with a 1% ointment of the compound are shown in Fig. 2. On the treated side the herpetic lesions have healed, the skin showing only residual trauma from scarification, whereas on the control side the lesions are in mid-course of evolution. Satisfactory cures were also obtained with VIb and acycloguanosine as a 1% solution in dimethylsulphoxide.

Metabolic disposition

Studies have been carried out to evaluate the absorption, metabolism, tissue distribution and excretion of 9-(2-hydroxyethoxymethyl)guanine in several animal species.

To evaluate the urinary and faecal excretion ^{14}C-acycloguanosine labelled at the 8-position of the guanine nucleus was given to mice either orally or subcutaneously. Absorption of the drug given by mouth was incomplete. In the 4-h urine sample 23% of the ^{14}C was recovered. In the 24- and 48-h urine samples less than half the given dose was recovered. Most of the remaining radioactivity was found in the faeces. In mice treated s.c. with ^{14}C-acycloguanosine (50 mg per kg body weight; 5.5×10^7 d.p.m. per mouse) 82% of the radioactivity was excreted in the urine by 4 h and 94% by 24 h; the faecal excretion was 2.3%. Over 95% of the radioactivity excreted in the urine over a 24-h period was recovered as the unmetabolised compound. Analysis of the urine by high-pressure liquid chromatography (HPLC) on an Aminex A-28 column eluted with 15 mM sodium acetate buffer, *p*H 4.1, showed a single major peak of ^{14}C radioactivity with a retention time of 50 min, corresponding to authentic acycloguanosine. All fractions from

©1978 Nature Publishing Group

the column were counted. There was practically no cleavage of the compound *in vivo*, since only traces of radioactive guanine and uric acid were found in the urine. A minor metabolite (about 1% of the radioactivity) which was highly retained on the liquid chromatography column was characterised as 9-carboxymethoxymethylguanine by comparing its retention time (390 min) with that of the synthetic compound.

To determine whether repeated administration of acycloguanosine to mice would increase its biotransformation, the drug was given daily for 7 d to a group of mice (50 mg per kg, s.c.). On the last day the mice received a dose of labelled compound. The HPLC profile of ^{14}C radioactivity in the 24-h urine of mice showed no alteration in the metabolism of the compound.

Administration of ^{14}C-acycloguanosine (50 mg per kg, s.c.) to groups of CD-1 mice gave the tissue distribution results shown in Fig. 3. In all tissues there was a rapid disappearance of the compound in the first 2 h after administration, followed by a slower disappearance over a 24-h period. The levels in the kidney, intestine, lung and liver were higher than in the blood. The concentration in the brain was significantly lower than in the blood and other tissues, but the compound was still detectable at 24 h. When ^{14}C-labelled compound was administered orally (50 mg per kg) to mice its concentration in the plasma at 30 min was 13.5 nmol ml^{-1}, but its disappearance from the plasma was slower than in the case of subcutaneous administration. There was no appreciable binding of acycloguanosine to plasma or to any tissue constituents. In an *in vitro* study of plasma binding ^{14}C-acycloguanosine was incubated with the plasma of CD-1 mice at 37 °C for 45 min in a Dubnoff shaking incubator. Plasma binding was determined by ultrafiltration.

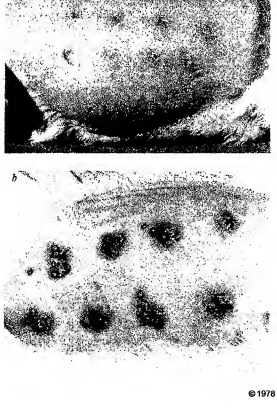


Fig. 2 Treatment of cutaneous herpes infection in a guinea-pig with 1% acycloguanosine ointment in a water-soluble base. *a*, Treated side; lesions healed after 3 days of treatment. *b*, Control side; lesions still in process of development.

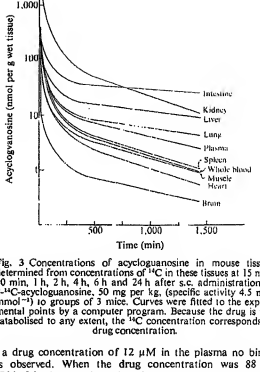


Fig. 3 Concentrations of acycloguanosine in mouse tissues determined from concentrations of ^{14}C in these tissues at 15 min, 30 min, 1 h, 2 h, 4 h, 6 h and 24 h after s.c. administration of 8-^{14}C-acycloguanosine, 50 mg per kg, (specific activity 4.5 mCi $mmol^{-1}$) to groups of 3 mice. Curves were fitted to the experimental points by a computer program. Because the drug is not catabolised to any extent, the ^{14}C concentration corresponds to drug concentration.

At a drug concentration of 12 μM in the plasma no binding was observed. When the drug concentration was 88 μM, 12.7% of the drug was bound to plasma.

In studies of the acute toxicity of acycloguanosine, the LD_{50} in mice given the drug orally was greater than 10,000 mg per kg whereas the LD_{50} in mice by the intraperitoneal route was 1,000 mg per kg. In 30-d studies in mice daily oral administration of 450 mg per kg of acycloguanosine produced no toxicity.

Related studies in rats and dogs on the metabolism, pharmacokinetics and toxicology of acycloguanosine will be described elsewhere; preliminary results are similar to those found in mice.

A study of the mechanism of the selective action of acycloguanosine has shown that it is phosphorylated by the herpes-specified thymidine kinase and converted to the triphosphate in herpes-infected cells to a much greater extent than in uninfected cells. Moreover, the triphosphate of acycloguanosine is more inhibitory to the viral DNA polymerase than to the α-DNA polymerase of the cell[12].

The higher antiviral activity in comparison with existing anti-herpes compounds, good activity in animal models, the very low toxicity and the lack of metabolic degradation on systemic administration make 9-(2-hydroxyethoxymethyl)guanine a promising candidate for clinical trials against the various manifestations of herpesvirus infection in man.

We thank Mr N. M. Oliver, Ms T. Creagh and Ms A. Little for technical assistance.

Received 19 September 1977; accepted 13 February 1978.

1. Bauer, D. J. *Ann. N.Y. Acad. Sci.* 130, 314–319 (1965).
2. Davies, W. L. *et al. Science* 144, 862–863 (1964).
3. Herrmann, E. C. *Proc. Soc. exp. Biol. Med.* 107, 142–145 (1961).
4. De Riedder, J. & Privat de Garilhe, M. *Antimicrob. Ag. Chemother.* 578–584 (1963).
5. Kaufman, H. E. & Heidelberger, C. *Science* 145, 585–586 (1964).
6. Elion, G. B., Rideout, J. L., de Miranda, P., Collins, P. & Bauer, D. J. *Ann. N.Y. Acad. Sci.* 255, 468–480 (1975).
7. Lin, T. S., Neuman, J. F., Cheng, Y. C., Prusoff, W. H. & Ward, D. C. *J. med. Chem.* 19, 495–498 (1976).
8. Wiskowski, J. T., Robins, R. K., Sidwell, R. W. & Simon, L. N. *J. med. Chem.* 15, 1150–1154 (1972).
9. Schaeffer, H. J., Gurwara, S., Vince, R. & Bittner, S. *J. med. Chem.* 14, 367–369 (1971).
10. Rasla, B., Blatkovic, D., Sorm, F. & Škoda, J. *Experientia* 16, 487 (1960).
11. Jones, B. R., Wise, J. B. & Patterson, A. *Evaluation of Drug Effects in the Eye*, 83–87 (ed. Pigott, P. V.) (Association of Medical Advisers to the Pharmaceutical Industry, London, 1968).
12. Corwin, M. E. *et al. Invest. Ophthalmol.* 2, 578–583 (1963).
13. Elion, G. B. *et al. Proc. natn. Acad. Sci. U.S.A.* 74, 5716–5720 (1977).

©1978 Nature Publishing Group

Downloaded By: [Derycz, Andrei] At: 23:26 21 July 2009

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(4–7), 271–285 (2001)

ACYCLIC/CARBOCYCLIC GUANOSINE ANALOGUES AS ANTI-HERPESVIRUS AGENTS

E. De Clercq, G. Andrei, R. Snoeck, L. De Bolle, L. Naesens, B. Degrève, J. Balzarini, Y. Zhang, D. Schols, P. Leyssen, C. Ying, and J. Neyts

Rega Institute for Medical Research, K. U. Leuven,
B-3000 Leuven, Belgium

ABSTRACT

Several guanosine analogues, i.e. acyclovir (and its oral prodrug valaciclovir), penciclovir (in its oral prodrug form, famciclovir) and ganciclovir, are widely used for the treatment of herpesvirus [i.e. herpes simplex virus type 1 (HSV-1), and type 2 (HSV-2), varicella-zoster virus (VZV) and/or human cytomegalovirus (HCMV)] infections. In recent years, several new guanosine analogues have been developed, including the 3-membered cyclopropylmethyl and -methenyl derivatives (A-5021 and synguanol) and the 6-membered D- and L-cyclohexenyl derivatives. The activity of the acyclic/carbocyclic guanosine analogues has been determined against a wide spectrum of viruses, including the HSV-1, HSV-2, VZV, HCMV, and also human herpesviruses type 6 (HHV-6), type 7 (HHV-7) and type 8 (HHV-8), and hepatitis B virus (HBV). The new guanosine analogues (i.e. A-5021 and D- and L-cyclohexenyl G) were found to be particularly active against those viruses (HSV-1, HSV-2, VZV) that encode for a specific thymidine kinase (TK), suggesting that their antiviral activity (at least partially) depends on phosphorylation by the virus-induced TK. Marked antiviral activity was also noted with A-5021 against HHV-6 and with D- and L-cyclohexenyl G against HCMV and HBV. The antiviral activity of the acyclic/carbocyclic nucleoside analogues could be markedly potentiated by mycophenolic acid, a potent inhibitor of inosine 5′-monophosphate (IMP) dehydrogenase. The new carbocyclic guanosine analogues (i.e. A-5021 and D- and L-cyclohexenyl G) hold great promise, not only as antiviral agents for the treatment of herpesvirus infections, but also an antitumor agents for the

Copyright © 2001 by Marcel Dekker, Inc. www.dekker.com

EXHIBIT 4

ORDER REPRINTS

combined gene therapy/chemotherapy of cancer, provided that (part of) the tumor cells have been transduced by the viral (HSV-1, VZV) TK gene.

INTRODUCTION: ACYCLOVIR, PENCICLOVIR AND GANCICLOVIR

Foremost among the most frequently used antiherpetic drugs are the guanosine analogues acyclovir (and its oral prodrug form, valaciclovir), penciclovir (under its oral prodrug form, famciclovir) and ganciclovir (which can be administered either intravenously or orally). These guanosine analogues have been pursued primarily for the treatment of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella-zoster virus (VZV) and human cytomegalovirus (HCMV) infections. Acyclovir has been the gold standard for the treatment of mucosal, cutaneous and systemic HSV-1 and HSV-2 infections (including herpes encephalitis and genital herpes) and VZV infections (varicella and herpes zoster), and can also be used in the prophylaxis of genital and orofacial HSV infections, and VZV and CMV infections (1). The L-valyl ester, valaciclovir, the oral prodrug of acyclovir, has been found to achieve substantially higher plasma levels of acyclovir than oral acyclovir itself; oral valaciclovir has proven to be particularly useful in the treatment of herpes zoster (2) and in the prevention of HCMV disease after renal transplantation (3). The indications for the use of famciclovir, the oral prodrug form of penciclovir (4), are identical to those of valaciclovir, *viz.* the treatment of HSV and VZV infections. The total systemic availability of penciclovir, following oral famciclovir, can be considered similar to that of acyclovir following oral valaciclovir. Upon intravenous administration, penciclovir and acyclovir afforded equivalent efficacy in the treatment of HSV infections in immunocompromised patients (5). Although ganciclovir is at least as effective, if not more so, than penciclovir and acyclovir against HSV-1 and HSV-2, it has been developed mainly for the treatment of HCMV infections (i.e., HCMV retinitis in AIDS patient) (6), where it can be administered either intravenously or orally (as mentioned above), or locally, as an intravitreal implant. Acyclovir was the first guanosine analogue described as an antiviral agent: it has served as the prototype for the development of a whole series of new acyclic or carbocyclic guanosine analogues, including penciclovir and ganciclovir, as well as a number of both three- and six-membered sugar derivatives (Fig. 1). The comparative antiviral potency, based on the 50% effective concentration (EC_{50}), of these compounds against the herpesviruses HSV-1, HSV-2, VZV and HCMV is presented in Table 1.

LOBUCAVIR

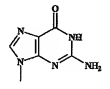
Lobucavir {1*R*(1α,2β,3α)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine}, which has also been referred to as (R)-BHCG, SQ-34,514, BMS-180194 and cygalovir, corresponds to the active enantiomer of (±)BHCG (SQ-33,054, cyclobut-G)

Downloaded By: [Dalcyc, Peter] At: 21:26 21 July 2009

Copyright © Marcel Dekker, Inc. All rights reserved.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS



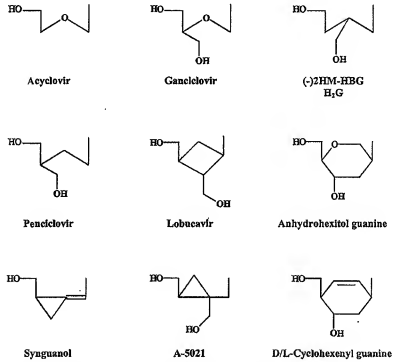


Figure 1. Structural formulae of carbocyclic and acyclic guanosine analogues.

(8,9), which was originally shown to possess broad-spectrum antiviral activity against several herpesviruses (i.e. HSV-1, HSV-2, VZV, HCMV,...) as well as HIV (21). The phosphorylation of lobucavir is initiated by the HSV- or VZV-encoded thymdine kinase (TK) (8), and the eventual action of lobucavir against HSV-1, HSV-2 and VZV is based upon inhibition of the viral DNA polymerase by the triphosphate of lobucavir (22). The triphosphate of lobucavir is also a potent inhibitor of the HCMV DNA polymerase (Ki: 5 nM) (23), although lobucavir inhibits HCMV replication only at a 100-fold higher concentration (Table 1) than that required to inhibit HSV-1, HSV-2 or VZV. The reason is that lobucavir is only

Downloaded By: [Decyc, Mcec] At: 23:26 21 July 2009

Copyright © Marcel Dekker, Inc. All rights reserved

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS

Table 1. Comparative Potency of Different Antiherpetic Compounds Against Different Herpesviruses

Compound	50% Effective Concentration EC_{50} (μM)				References
	HSV-1	HSV-2	VZV	HCMV	
Acyclovir	+++	+++	++	+	1,7
Penciclovir	+++	++	++	(+)	4
Ganciclovir	+++++	+++++	++	++	6,7
Lobucavir	++++	++++	+++	++	8,9
H2G	++++	++++	++++	(+)	§
A-5021	++++	+++	+++	+	10, this report
Synguanol	+	–	+	++	11,12
Anhydrohexitol G	++(+)	+++(+)	++	++	13,14
D-Cyclohexenyl G	++++	+++	++	++	15
L-Cyclohexenyl G	+++(+)	+++	++	++	15
References	16,17	16,18	19	20	

§E. De Clercq, unpublished data.
*–, if $EC_{50} > 100\ \mu M$.
+, if $EC_{50} = 10–100\ \mu M$.
++, if $EC_{50} = 1–10\ \mu M$.
+++, if $EC_{50} = 0.1–1\ \mu M$.
++++, if $EC_{50} = 0.01–0.1\ \mu M$.
+++++, if $EC_{50} = 0.001–0.01\ \mu M$.
( ) if only borderline, or if not accurately determined.

Downloaded By: [Decyx, Rene] At: 23:26 21 July 2009

weakly phosphorylated in HCMV-infected cells, this phosphorylation being independent of the UL97 protein kinase (the HCMV-encoded phosphotransferase that is responsible for the phosphorylation of ganciclovir) (23).

H2G

H2G corresponds to (-)2HM-HBG or the (-)enantiomer of 9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine. Like acyclovir, penciclovir and lobucavir, H2G is primarily active against HSV-1, HSV-2 and VZV (24). H2G has been found to suppress simian varicella virus infection in African green monkeys at a dose as low as 1 mg/kg/day (25) and has been pursued for the treatment of VZV infections in humans.

A-5021

A new class of compounds, based on the cycloprophylmethyl entity as the sugar substitute, namely A-5021 or (1′*S*,2′*R*)-9-[[1′,2′-bis(hydroxymethyl)cyclorop-1′-yl]methyl]guanine, has been recently identified as potent and selective

Copyright © Marcel Dekker, Inc. All rights reserved.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS

inhibitors of HSV-1, HSV-2 and VZV (10). A-5021 proved even more active against HSV-1, HSV-2 and VZV than either acyclovir or penciclovir (Table 1), and, as for acyclovir and penciclovir, little activity was noted against HCMV. Again, as for acyclovir and penciclovir, the anti-HSV and anti-VZV activity of A-5021 was found to depend on the phosphorylation by the HSV- and VZV-encoded TK. The intracellular half-life of A-5021 triphosphate was longer than that of acyclovir triphosphate but shorter than that of penciclovir triphosphate. Inhibition of the viral DNA polymerase was strongest with acyclovir triphosphate, followed by A-5021 triphosphate and then penciclovir triphosphate (26). Thus, in comparison with the triphosphates of acyclovir and penciclovir, A-5021 triphosphate accumulates for a longer time in the infected cells than acyclovir triphosphate and effects a stronger inhibition of the viral DNA polymerase than penciclovir triphosphate. In several murine models of HSV infection, A-5021 has proven more effective than acyclovir and penciclovir, i.e. with regard to reduction in the severity of herpetic skin lesions and protection against herpetic encephalitis (27).

SYNGUANOL

Synguanol corresponds to (Z)-2-(hydroxymethyl)cyclopropylidene)methylguanine. Synguanol is particularly active against HCMV (EC_{50}: 0.04–2.0 μM, depending on the assay system) (11,12); it has also marked activity against Epstein-Barr virus (EBV) but only limited potency against HSV-1, HSV-2 and VZV (11). The antiviral potency of synguanol is enantioselective. This enantioselectivity likely reflects differences in the rates of intracellular phosphorylation and/or affinities of the corresponding triphosphates for the target viral DNA polymerases (28). In addition to synguanol, various other (Z)-2-(hydroxymethyl)cyclopropylidene) methylpurines and–pyrimidines have been described as antiviral agents (i.e. synadenol, syncytol, synthymol, so named in analogy with synguanol) (29). Synguanol, as well as other methylenecyclopropane analogues of nucleosides, have proven effective in the treatment of murine cytomegalovirus (MCMV) infections in mice (30).

ANHYDROHEXITOL G

Various 1,5-anhydrohexitol nucleoside analogues have been found to exhibit marked activity against HSV-1, HSV-2, VZV and HCMV, the most active congeners being those with guanine (13), 5-iodouracil (13), 5-ethyluracil (14) or 5-trifluoromethyluracil (31) as the base moiety. Their antiviral activity must depend, at least partially, on a specific phosphorylation by the virus-encoded TK, since these compounds are less active against TK-deficient mutants of HSV-1. As HCMV does not encode for a virus-specific thymidine kinase, the activity noted with 1,5-anhydrohexitol guanine against HCMV would suggest that the compound must be phosphorylated by the UL97 protein kinase, or another phosphotransferase, in the HCMV-infected cells.

Downloaded By: [Dezyra, Peter] At: 23:26 21 July 2009

Copyright © Marcel Dekker, Inc. All rights reserved.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS

D- AND L-CYCLOHEXENYL G

The cyclohexene nucleoside analogues can be considered as analogues of the natural furanose nucleosides where the ring oxygen has been replaced by a double bond. D-cyclohexenyl G and L-cyclohexenyl G were found to possess similar potency (15) against HSV-1, HSV-2, VZV and CMV (Table 1). This is not the case for the D- and L-anhydrohexitol nucleoside analogues where only the D-analogues demonstrated antiviral activity (32). As the activity of D- and L-cyclohexenyl G was lower against TK-deficient (TK^-) HSV-1 strains that against TK^+ strains, the virus-induced phosphorylation may be an important determinant in their anti-HSV activity. In fact, both enantiomers of cyclohexenyl G could be readily accommodated in the active site of the viral TK (33). D- and L-cyclohexenyl guanine represent the first example of a broad-spectrum anti-herpesvirus compound exhibiting similar activity in their L- and D-form for which an explanation can be offered at the molecular (i.e. viral TK) level (33).

ANTIVIRAL ACTIVITY SPECTRUM OF ACYCLIC/CARBOCYCLIC GUANOSINE ANALOGUES

In comparison with acyclovir, the "gold standard" for the chemotherapy of HSV and VZV infections, the D- and L-cyclohexenyl guanines (15) were slightly more potent against HSV-1 and slightly less potent against HSV-2. Also A-5021 has proven to be more potent than acyclovir against both HSV-1 and HSV-2 (10). In our investigations we found D- and L-cyclohexenyl G to be equipotent with acyclovir, and A-5021 to be 20-fold more potent than acyclovir, in inhibiting VZV replication (Table 2). The virus-encoded thymidine kinase clearly contributed to the activity

Table 2. Activity of Guanosine Analogues Against Various Strains of Varicella-Zoster Virus (VZV) in Human Embryonic Lung (HEL) Cells

	EC_{50} (μg/ml)[a]			
	TK^+ VZV		TK^- VZV	
Compound	YS	OKA	07/1	YS/R
Acyclovir	1.1	0.8	13	28
Penciclovir	0.6	0.6	8	29
A-5021	0.04	0.05	3	16
D-Cyclohexenyl G	0.5	0.6	2.1	2.8
L-Cyclohexenyl G	1.2	1.9	5.8	6.8

[a] 50% Effective concentration, or concentration required to reduce virus plaque formation by 50%. Virus input was 20 plaque forming units (PFU). The compounds did not show a microscopically detectable alteration of normal cell morphology or inhibition of host cell growth at the highest concentration tested (50 μg/ml), except for D- and L-cyclohexenyl G that inhibited HEL cell growth by 50% at a concentration of 11 and 38 μg/ml, respectively.

Downloaded By: [Decyx, Peter] At: 23:26 21 July 2005

Copyright © Marcel Dekker, Inc. All rights reserved.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS

Table 3. Activity of Guanosine Analogues Against Various Strains of Human Cytomegalovirus (HCMV) in Human Embryonic Lung (HEL) Cells

	EC_{50} (μg/ml)[a]			
Compound	AD-169	Davis	Ly 9990	U 9070
Acyclovir	16	16	...	...
Penciclovir	>50	>50	...	...
Ganciclovir	0.77	0.62	7.5	10.8
A-5021	8	12	...	...
D-Cyclohexenyl G	0.47	0.83	0.38	0.13
L-Cyclohexenyl G	1.05	3.97	0.79	0.4

[a]50% Effective concentration, or concentration required to reduce virus plaque formation by 50%. Virus input was 100 plaque forming units (PFU). The compounds did not show a microscopically detectable alteration of normal cell morphology or inhibition of host cell growth at the highest concentration tested (50 μg/ml), except for D- and L-cyclohexenyl G that inhibited HEL cell growth by 50% at a concentration of 11 and 38 μg/mL, respectively.

of the compounds against VZV, since they were less active against TK^- than TK^+ VZV strains (Table 2).

Like acyclovir, A-5021 did not shown much activity against HCMV (Table 3). However, both D- and L-cyclohexenyl G proved active against HCMV at roughly the same concentration as ganciclovir, and, in addition, D- and L-cyclohexenyl retained marked activity against HCMV strains that were resistant to ganciclovir (Table 3).

Marked inhibitory activity was noted with A-5021 against both human herpesvirus type 6 strains HHV-6A and HHV-6B (Table 4): it was about 10-fold more

Table 4. Activity of Guanosine Analogues Against Human Herpesvirus (HHV-6) in Human Lymphoblast Cell Lines

	EC_{50} (μg/ml)[a]			
	HHV-6A (GS) in HSB-2 Cells		HHV-6B (Z-29) in MOLT-3 Cells	
Compound	CPE	DNA Synthesis	CPE	DNA Synthesis
Acyclovir	27	41	36	41
Penciclovir	71	85	>100	>100
Ganciclovir	10	8	16	15
Labucavir	2.17	1.96	2.36	4.85
A-5021	2.64	3.45	3.39	3.48

[a]50% Effective concentration, or concentration required to reduce virus-induced CPE (cytopathic effect, determined microscopically) or viral DNA synthesis (determined by hybridisation with a digoxigenin-labelled probe) by 50%. The minimum cytotoxic concentrations, causing a microscopically detectable alteration of normal cell morphology, were 100 μg/ml (acyclovir), $\geq$100 μg/ml (penciclovir), 20 μg/ml (ganciclovir), 10 μg/ml (lobucavir) and $\geq$50 μg/ml (A-5021).

Downloaded By: [Decyca, Nicole] At: 23:26 21 July 2009

Copyright © Marcel Dekker, Inc. All rights reserved.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS

potent than acyclovir. A-5021 was about equipotent as lobucavir, but, as it was less toxic to the host cells (Table 4, footnote), it achieved a greater selectivity index against HHV-6 than lobucavir.

A-5021 did not inhibit the replication of human herpesvirus type 7 (HHV-7) in human T-lymphocyte (sup T1) cells at the highest (subtoxic) concentration tested (60 μg/ml). Nor did D/L-cyclohexenyl G (20 μg/ml), acyclovir (250 μg/ml), penciclovir (170 μg/ml), ganciclovir (50 μg/ml) or H2G (100 μg/ml). Lobucavir proved active against HHV-7 at an EC_{50} of 5 μg/ml, that is a concentration that was 3-fold lower than its CC_{50} (50% cytotoxic concentration).

A-5021 was also evaluated for its activity against Epstein-Barr virus (EBV) and human herpesvirus type 8 (HHV-8), in P3HR-1 and BCBL-1 cells, respectively; anti-EBV and anti-HHV-8 activity was assessed by monitoring viral DNA synthesis with digoxigenin-labelled probes specific for EBV and HHV-8. A-5021 inhibited EBV DNA synthesis at an EC_{50} of 1.0 μg/ml (as compared to 1.4 μg/ml for acyclovir). Neither A-5021 nor acyclovir inhibited HHV-8 at a concentration of 20 μg/ml.

The guanosine analogues acyclovir, penciclovir, ganciclovir, lobucavir, synguanol and A-5021 were also evaluated against a member of the Flaviviridae, namely Yellow fever virus [as a surrogate substitute for hepatitis C virus (HCV)], but as expected, no activity was found with any of the compounds at concentrations up to 200 μg/ml.

When the guanosine analogues were evaluated against hepatitis B virus (HBV), marked activity was noted for lobucavir and D/L-cyclohexenyl G (Table 5). The latter was about 10-fold more potent against HBV than penciclovir, which, in its prodrug form (famciclovir), has been the subject of clinical studies in HBV-infected patients. A-5021 and the other guanosine analogues that were tested did not show appreciable activity against HBV. Those that did, inhibited the replication of wild-type HBV (Hep AD38 cells) and lamivudine-resistant HBV, containing the methionine → valine substitution (M550V) in the DNA polymerase (34), at similar EC_{50} values (Table 5).

ENHANCEMENT OF THE ANTIVIRAL ACTIVITY OF ACYCLIC/CARBOCYCLIC GUANOSINE ANALOGUES BY MYCOPHENOLATE MOFETIL

Mycophenolate mofetil (which is currently used as an immunosuppressant in kidney transplant recipients) is the morpholinoethyl ester of mycophenolic acid (Fig. 2). The latter is known to be a potent inhibitor of IMP dehydrogenase (35). Like other IMP dehydrogenase inhibitors (Fig. 2), such as ribavirin, tiazofurin, EICAR (36) and VX-497 (37), mycophenolic acid is expected to reduce the intracellular pool levels of GTP and dGTP through its inhibitory effect on the conversion of IMP to XMP (Fig. 3). Through depletion of the intracellular dGTP pools, mycophenolic acid may then facilitate the effectiveness of the triphosphates of the acyclic guanosine analogues (acyclovir, penciclovir, ganciclovir, etc.) in their competitive

Downloaded By: [Derycke, Peter] At: 23:26 21 July 2009

Copyright © Marcel Dekker, Inc. All rights reserved.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS

Table 5. Activity of Guanosine Analogues Against Hepatitis B Virus (HBV) in Hep AD38 and Hep AD79 Cells

	EC_{50} (μg/ml)[a]	
Compound	Hep AD38	Hep AD79
Acyclovir	>50	>50
Penciclovir	46	45
Ganciclovir	>50	>50
Lobucavir	0.5	1.1
Synguanol	>100	>100
A-5021	>20	>20
D/L-Cyclohexenyl G	6	3
Adefovir (PMEA)	0.05	0.14
Tenofovir (PMPA)	0.05	0.05
Lamivudine (3TC)	0.0037	0.82

[a]50% Effective concentration, or concentration required to reduce viral DNA synthesis (as monitored by hybridisation with a digoxigenin-labelled probe) by 50%. Hep AD38 and Hep AD79 cells represent hepatoma cells transfected with a cDNA copy of the pregenomic RNA of wild-type virus or mutant virus (containing the M550V mutation in the DNA polymerase, which leads to resistance to 3TC), respectively. Data taken from reference 34; and unpublished data (C. Ying, J. Neyts and E. De Clercq).

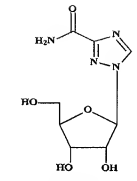
inhibitory effect (with respect to dGTP) at the viral DNA polymerase level. Mycophenolic acid was found to markedly (up to 100-fold) potentiate the inhibitory effects of acyclovir, penciclovir and ganciclovir on HSV-1, HSV-2, VZV and CMV *in vitro* (Table 6) and *in vivo* (38); and a similar marked enhancement was noted for the activity of H2G against HSV-1, HSV-2 and both TK^+ and TK^- VZV (39). In hairless mice infected intracutaneously with HSV-1, combined use of systemic acyclovir (20 mg/kg/day) and topical mycophenolate mofetil (5%) resulted in an almost complete protection, whereas single use of either compound had virtually no protective effect (40). Mycophenolic acid has also been found to markedly potentiate the activity of lobucavir against HSV-1, HSV-2, TK^- HSV-1 and HCMV: for TK^-HSV-1 the 50% effective concentration (EC_{50}) of lobucavir was decreased up to 1400-fold upon combination with mycophenolic acid (41). Similarly, mycophenolic acid brought about a significant enhancement (up to 200-fold) of the activity of A-5021 against HSV-1, HSV-2 and TK^- HSV-1 [J. Neyts and E. De Clercq (unpublished data, 1999)]. These observations may have important implications in transplant recipients, that under therapy with mycophenolate mofetil, develop opportunistic herpesvirus infections that are amenable to treatment with any of the acyclic guanosine analogues mentioned above (acyclovir, penciclovir, ganciclovir, etc.). It would now seem imperative to examine whether mycophenolate mofetil also potentiates the antiviral effects of the other guanosine analogues, i.e., synguanol, anhydrohexitol guanine and L- and D-cyclohexenyl guanine.

Downloaded By: [Neyts, Johan] At: 23:26 21 July 2009

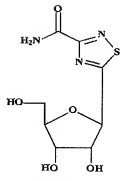
Copyright © Marcel Dekker, Inc. All rights reserved.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

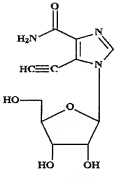
ORDER REPRINTS



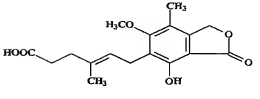
Ribavirin



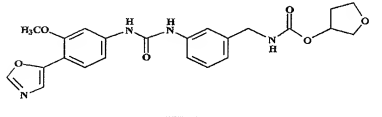
Tiazofurin



EICAR



Mycophenolic acid



VX-497

Figure 2. Mycophenolic acid and other IMP dehydrogenase inhibitors (ribavirin, tiazofurin, EICAR and VX-497).

Downloaded By: [Dezyre, Retec] At: 23:26 21 July 2009

Copyright © Marcel Dekker, Inc. All rights reserved

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS

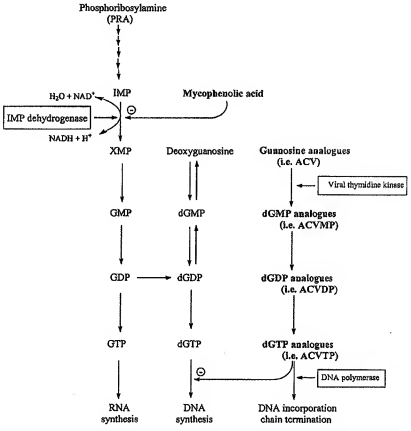


Figure 3. Interaction of mycophenolic acid (*via* inhibition of IMP dehydrogenase) with inhibitory effects of the guanosine analogues (triphosphates) at the DNA polymerase level.

ACYCLIC/CARBOCYCLIC GUANOSINE ANALOGUES IN THE COMBINED GENE THERAPY/CHEMOTHERAPY OF CANCER

As originally demonstrated 15 years ago (42), transformation of tumor cells, i.e. murine mammary FM3A carcinoma cells, with the HSV-1 thymidine kinase (TK) gene makes them highly sensitive to the cytostatic action of all those anti-herpesvirus agents (including ganciclovir) that for their antiviral activity rely upon phosphorylation by the viral TK. For ganciclovir, this cytostatic activity results from the incorporation of the compound (following its conversion to the triphosphate) into the DNA of the HSV-1 (or HSV-2) TK gene-transfected cells (43). Like ganciclovir, various other guanosine analogues also become significantly more cytostatic to tumor cells, i.e. murine mammary carcinoma (FM3A) cells, after these

Downloaded By: [Dezyra, Peter] At: 23:26 21 July 2009

Copyright © Marcel Dekker Inc. All rights reserved.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS

Table 6. Potentiating Effect of Mycophenolic Acid (MPA) on the Anti-herpesvirus Activity of Guanosine Analogues

	EC_{50} (μg/ml)[a]					
	HSV-1		HSV-2		TK⁻ HSV-1	
Compound	−MPA	+MPA	−MPA	+MPA	−MPA	+MPA
Acyclovir	5.3	0.1	2.6	0.04	56	0.3
Penciclovir	6.6	0.5	5.3	0.7	>100	2.6
Ganciclovir	1.0	0.01	1.4	0.05	18	0.4
Lobucavir	1.8	0.03	0.7	0.004	11	0.06
H2G	5.3	0.2	11	0.2	>100	0.08
A-5021	1.3	0.006	1.5	0.08	≥70	1.3

[a]50% Effective concentration, or concentration required to reduce virus-induced cytopathicity in Vero cells by 50%.
MPA was used at a concentration of 2.5 μg/ml, at which it had by itself no antiviral effect.
Data taken from references 38, 39 and 41; and unpublished data (J. Neyts and E. De Clercq).

cells have been transfected by the HSV-1 TK gene (44). These observations have been extended to human osteosarcoma cells transfected by the HSV-1 TK/GFP fusion gene (45) (Table 7). A-5021 and D/L-cyclohexenyl guanine, but not synguanol, exhibited potent cytostatic activity against OST/TK⁻ HSV-1 TK $(GFP)^+$ cells, as

Table 7. Inhibitory Effects of Different Guanosine Analogues on the Proliferation of HSV Thymidine Kinase (TK) Gene-Transfected Tumor Cells

	EC_{50} (μM)[a]		
Compound	OST/TK⁻	OST/TK⁻ HSV-1 TK $(GFP)^+$	S.I.[b]
Acyclovir	73 ± 29	0.059 ± 0.015	1,237
Penciclovir	231 ± 13	0.013 ± 0.002	17,769
Ganciclovir	44 ± 22	0.001 ± 0.0005	44,000
Lobucavir	18 ± 0.4	0.008 ± 0.0001	2,250
H2G	>250	0.009 ± 0.003	>27,778
A-5021	137 ± 10	0.0006 ± 0.0002	228,333
Synguanol	13 ± 4	14 ± 3.3	0.9
D/L-Cyclohexenyl G	3.2 ± 0.5	0.005 ± 0.004	640

[a]50% Effective concentration, or concentration required to inhibit cell proliferation by 50%. OST/TK: human osteosarcoma cells deficient in cytosol TK; OST/TK⁻ HSV-1 TK(GFP)⁺: OST/TK⁻ cells transduced by the HSV-1 TK GFP (green fluorescence protein) fusion gene.
[b]Selectivity index, or ratio of EC_{50} (OST/TK⁻) to EC_{50} [OST/TK⁻ HSV-1 TK $(GFP)^+$].
Data taken from reference 45; and unpublished data (B. Degrève, E. De Clercq and J. Balzarini).

Downloaded By: [Dezyra, Peter] At: 23:26 21 July 2009

Copyright © Marcel Dekker, Inc. All rights reserved

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS

did H2G, lobucavir and ganciclovir. Of all the compounds tested, A-5021 showed the highest ratio (about 200,000) in the EC_{50} for non-transfected *versus* the EC_{50} for transfected osteosarcoma cells. The acyclic/carbocyclic guanosine analogues could thus be envisaged as potent anticancer agents, provided the tumor cells are engineered in such a way that they express the viral (HSV-1) thymidine kinase. It is not even necessary to transform all the tumor cells, since the guanosine analogues are endowed with a marked bystander killing effect, i.e. they are able to kill up to 90% of the cells of a population that contain only 10% viral TK gene-transfected tumor cells (45).

CONCLUSION

The most novel congeners among the carbocyclic guanosine analogues contain either a 3- or 6-membered sugar substitute: *viz.* the cyclopropylmethyl derivative A-5021 and D- and L-cyclohexenyl guanine. These new guanosine analogues were found to possess an extended activity spectrum as compared to that of acyclovir. While equally active (D- and L-cyclohexenyl guanine) or more active (A-5021) than acyclovir against HSV-1, HSV-2 and VZV, D- and L-cyclohexenyl guanine showed marked activity against HCMV, including HCMV strains that were resistant to ganciclovir. A-5021 was quite effective against HHV-6, as so was D/L-cyclohexenyl guanine against HBV. Combination with mycophenolic acid (MPA) markedly potentiated the antiviral activity of the new guanosine analogues (i.e., A-5021), as it did with the older congeners (acyclovir, ganciclovir and penciclovir). Given their dependence on phosphorylation by the virus (i.e. HSV-1)-encoded thymidine kinase (TK), all these guanosine analogues, and particularly A-5021 and D- and L-cyclohexenyl guanine, should also be pursued from combined gene therapy/chemotherapy viewpoint, based on the observation that transduction of the tumor cells by the viral TK make them exqusitely sensitive to the cytostatic action of these antiviral compounds.

ACKNOWLEDGMENTS

Erik De Clercq holds the Prof. P. De Somer Chair for Microbiology at the Katholieke Universiteit Leuven and acknowledges the proficient editorial assistance of Mrs. Christiane Callebaut in the preparation of this manuscript. The original observations reported here were supported by grants from the Fonds voor Wetenschappelijk Onderzoek (FWO) – Vlaanderen (krediet G.0104.98), the Belgian Federation against Cancer and the Geconcerteerde Onderzoeksacties (Vlaamse Gemeenschap) (contract 2000/12). L. De Bolle is a research assistant and Johan Neyts a post-doctoral fellow from the "Fonds voor Wetenschappelijk Onderzoek – Vlaanderen (FWO)". B. Degrève and P. Leyssen are research assistants from the "Instituut voor Wetenschap & Technologie (IWT)".

Downloaded By: [Dezyck, Peter] At: 23:26 21 July 2009

Copyright © Marcel Dekker, Inc. All rights reserved.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS

REFERENCES

1. O'Brien, J.J.; Campoli-Richards, D.M. *Drugs*, **1989**, *37*, 233–309.
2. Beutner, K.R.; Friedman, D.J.; Forszpaniak, A.; Andersen, P.L.; Wood, M.J. *Antimicrob Agents Chemother.*, **1995**, *39*, 1546–1553.
3. Lowance, D.; Neumayer, H.-H.; Legendre, C.M.; Squifflet, J.-P.; Kovarik, J.; Brennan, P.J.; Norman, D.; Mendez, R.; Keating, M.R.; Coggon, G.L.; Crisp, A.; Lee, I.C. *N. Engl. J. Med.*, **1999**, *340*, 1462–1470.
4. Vere Hodge, R.A. *Antiviral Chem. Chemother.*, **1993**, *4*, 67–84.
5. Lazarus, H.M.; Belanger, R.; Candoni, A.; Aoun, M.; Jurewicz, R.; Marks, L.; The Penciclovir Immunocompromised Study Group. *Antimicrob. Agents Chemother.*, **1999**, *43*, 1192–1197.
6. Markham, A.; Faulds, D. *Drugs*, **1994**, *48*, 455–484.
7. Neyts, J.; Andrei, G.; Snoeck, R.; Jähne, G.; Winkler, I.; Helsberg, M.; Balzarini, J.; De Clercq, E. *Antimicrob. Agents Chemother.*, **1994**, *38*, 2710–2716.
8. Yamanaka, G.; Tuomari, A.V.; Hagen, M.; McGeever-Rubin, B.; Terry, B.; Haffey, M.; Bisacchi, G.S.; Field, A.K. *Mol. Pharmacol.*, **1991**, *40*, 446–453.
9. Maruyama, T.; Hanai, Y.; Sato, Y.; Snoeck, R.; Andrei, G.; Hosoya, M.; Balzarini, J.; De Clercq, E. *Chem. Pharm. Bull.*, **1993**, *41*, 516–521.
10. Iwayama, S.; Ono, N.; Ohmura, Y.; Suzuki, K.; Aoki, M.; Nakazawa, H.; Oikawa, M.; Kato, T.; Okunishi, M.; Nishiyama, Y.; Yamanishi, K. *Antimicrob. Agents Chemother.*, **1998**, *42*, 1666–1670.
11. Qiu, Y.-L.; Ksebati, M.B.; Ptak, R.G.; Fan, B.Y.; Breitenbach, J.M.; Lin, J.-S.; Cheng, Y.-C.; Kern, E.R.; Drach, J.C.; Zemlicka, J. *J. Med. Chem.*, **1998**, *41*, 10–23.
12. Rybak, R.J.; Hartline, C.B.; Qiu, Y.-L.; Zemlicka, J.; Harden, E.; Marshall, G.; Sommadossi, J.-P.; Kern, E.R. *Antimicrob. Agents Chemother.*, **2000**, *44*, 1506–1511.
13. Verheggen, I.; Van Aerschot, A.; Toppet, S.; Snoeck, R.; Janssen, G.; Balzarini, J.; De Clercq, E.; Herdewijn, P. *J. Med. Chem.*, **1993**, *36*, 2033–2040.
14. Verheggen, I.; Van Aerschot, A.; Van Meervelt, L.; Rozenski, J.; Wiebe, L.; Snoeck, R.; Andrei, G.; Balzarini, J.; Claes, P.; De Clercq, E. *J. Med. Chem.*, **1995**, *38*, 826–835.
15. Wang, J.; Froeyen, M.; Hendrix, C.; Andrei, G.; Snoeck, R.; De Clercq, E.; Herdewijn, P. *J. Med. Chem.*, **2000**, *43*, 736–745.
16. Andrei, G.; Snoeck, R.; Goubau, P.; Desmyter, J.; De Clercq, E. *Eur. J. Clin. Microbiol. Infect. Dis.*, **1992**, *11*, 143–151.
17. Andrei, G.; Snoeck, R.; De Clercq, E. *Antimicrob. Agents Chemother.*, **1995**, *39*, 1632–1635.
18. Andrei, G.; Snoeck, R.; De Clercq, E. *Antiviral Chem. Chemother.*, **1997**, *8*, 457–461.
19. Andrei, G.; Snoeck, R.; Reymen, D.; Liesnard, C., Goubau, P.; Desmyter, J.; De Clercq, E. *Eur. J. Clin. Microbiol. Infect. Dis.*, **1995**, *14*, 318–328.
20. Andrei, G.; Snoeck, R.; Schols, D.; Goubau, P.; Desmyter, J.; De Clercq, E. *Eur. J. Clin. Microbiol. Infect. Dis.*, **1991**, *10*, 1026–1033.
21. Norbeck, D.W.; Kern, E.; Hayashi, S.; Rosenbrook, W.; Sham, H.; Herrin, T.; Plattner, J.J.; Erickson, J.; Clement, J.; Swanson, R.; Shipkowitz, N.; Hardy, D.; Marsh, K.; Arnett, G.; Shannon, W.; Broder, S.; Mitsuya, H. *J. Med. Chem.*, **1990**, *33*, 1281–1285.
22. Terry, B.J.; Cianci, C.W.; Hagen, M.E. *Mol. Pharmacol.*, **1991**, *40*, 591–596.
23. Tenney, D.J.; Yamanaka, G.; Voss, S.M.; Cianci, C.W.; Tuomari, A.V.; Sheaffer, A.K.; Alam, M.; Colonno, R.J. *Antimicrob. Agents Chemother.*, **1997**, *41*, 2680–2685.

Downloaded By: [Dezyra, Peter] At: 21:26 21 July 2009

Copyright © Marcel Dekker, Inc. All rights reserved

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

ORDER REPRINTS

24. Abele, G.; Cox, S.; Bergman, S.; Lindborg, B.; Vissgarden, A.; Karlström, A.; Harmenberg, J.; Wahren, B. *Antiviral Chem. Chemother.*, **1991**, *2*, 163–169.
25. Soike, K.F.; Bohm, R.; Huang, J.-L.; Öberg, B. *Antimicrob. Agents Chemother.*, **1993**, *37*, 1370–1372.
26. Ono, N.; Iwayama, S.; Suzuki, K.; Sekiyama, T.; Nakazawa, H.; Tsuji, T.; Okunishi, M.; Daikoku, T.; Nishiyama, Y. *Antimicrob. Agents Chemother.*, **1998**, *42*, 2095–2102.
27. Iwayama, S.; Ohmura, Y.; Suzuki, K.; Ono, N.; Nakazawa, H.; Aoki, M.; Tanabe, I.; Sekiyama, T.; Tsuji, T.; Okunishi, M.; Yamanishi, K.; Nishiyama, Y. *Antiviral Res.*, **1999**, *42*, 139–148.
28. Zemlicka, J.; Qiu, Y.-L.; Kira, T.; Gullen, E.; Cheng, Y.-C.; Ptak, R.G.; Breitenbach, J.M.; Drach, J.C.; Hartline, C.B.; Kern, E.R. Abstracts of the Twelfth International Conference on Antiviral Research, Jerusalem, Israel. *Antiviral Res.*, **1999**, *41*, A34, no 10.
29. Qiu, Y.-L.; Breitenbach, J.M.; Lin, J.S.; Cheng, Y.-C.; Kern, E.R.; Drach, J.R.; Zemlicka, J. *Antiviral Chem. Chemother.*, **1998**, *9*, 341–352.
30. Rybak, R.J.; Zemlicka, J.; Qiu, Y.-L.; Hartline, C.B.; Kern, E.R. *Antiviral Res.*, **1999**, *43*, 173–188.
31. Ostrowski, T.; Wroblowski, B.; Busson, R.; Rozenski, J.; De Clercq, E.; Bennett, M.S.; Champness, J.N.; Summers, W.C.; Sanderson, M.R.; Herdewijn, P. *J. Med. Chem.*, **1998**, *41*, 4343–4353.
32. Andersen, M.W.; Daluge, S.M.; Kerremans, K.; Herdewijn, P. *Tetrahedron Lett.*, **1996**, *37*, 8147–8150.
33. Herdewijn, P. *Int. Antiviral News*, **1999**, *7*, 158–163.
34. Ying, C.; De Clercq, E.; Nicholson, W.; Furman, P.; Neyts, J. *J. Viral Hepatitis*, **2000**, *7*, 161–165.
35. Sintchak, M.D.; Fleming, M.A.; Futer, O.; Raybuck, S.A.; Chambers, S.P.; Caron, P.R.; Murcko, M.A.; Wilson, K.P. *Cell*, **1996**, *85*, 921–930.
36. De Clercq, E.; Cools, M.; Balzarini, J.; Snoeck, R.; Andrei, G.; Hosoya, M.; Shigeta, S.; Ueda, T.; Minakawa, N.; Matsuda, A. *Antimicrob. Agents Chemother.*, **1991**, *35*, 679–684.
37. Kwong, A.D. Abstracts of the Twelfth International Conference on Antiviral Research, Jerusalem, Israel. Antiviral Res., **1999**, *41*, A38, no 18.
38. Neyts, J.; Andrei, G.; De Clercq, E. *Antimicrob. Agents Chemother.*, **1998**, *42*, 216–222.
39. Neyts, J.; Andrei, G.; De Clercq E. *Antimicrob. Agents Chemother.*, **1998**, *42*, 3285–3289.
40. Neyts, J.; De Clercq E. *Antiviral Res.*, **1998**, *40*, 53–56.
41. Neyts J.; De Clercq, E. *Transplantation*, **1999**, *67*, 760–764.
42. Balzarini, J.; De Clercq, E.; Ayusawa, D.; Seno, T. *FEBS Lett.*, **1985**, *185*, 95–100.
43. Balzarini, J.; Bohman, C.; De Clercq, E. *J. Biol. Chem.*, **1993**, *268*, 6332–6337.
44. Balzarini, J.; Bohman, C.; Walker, R.T.; De Clercq, E. *Mol. Pharmacol.*, **1994**, *45*, 1253–1258.
45. Degrève, B.; De Clercq, E.; Balzarini, J.; *Gene Ther.*, **1999**, *6*, 162–170.

Downloaded By: [Neyts, Peter] At: 23:26 21 July 2009

Copyright © Marcel Dekker, Inc. All rights reserved.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

Request Permission or Order Reprints Instantly!

Interested in copying and sharing this article? In most cases, U.S. Copyright Law requires that you get permission from the article's rightsholder before using copyrighted content.

All information and materials found in this article, including but not limited to text, trademarks, patents, logos, graphics and images (the "Materials"), are the copyrighted works and other forms of intellectual property of Marcel Dekker, Inc., or its licensors. All rights not expressly granted are reserved.

Get permission to lawfully reproduce and distribute the Materials or order reprints quickly and painlessly. Simply click on the "Request Permission/Reprints Here" link below and follow the instructions. Visit the U.S. Copyright Office for information on Fair Use limitations of U.S. copyright law. Please refer to The Association of American Publishers' (AAP) website for guidelines on Fair Use in the Classroom.

The Materials are for your personal use only and cannot be reformatted, reposted, resold or distributed by electronic means or otherwise without permission from Marcel Dekker, Inc. Marcel Dekker, Inc. grants you the limited right to display the Materials only on your personal computer or personal wireless device, and to copy and download single copies of such Materials provided that any copyright, trademark or other notice appearing on such Materials is also retained by, displayed, copied or downloaded as part of the Materials and is not removed or obscured, and provided you do not edit, modify, alter or enhance the Materials. Please refer to our Website User Agreement for more details.

Order now!

Reprints of this article can also be ordered at
http://www.dekker.com/servlet/product/DOI/101081NCN100002298

Downloaded By: [Dargis, Peter] At: 21:56 23 July 2009

Acyclic Guanosine Analogs Inhibit DNA Polymerases α, δ, and ε with Very Different Potencies and Have Unique Mechanisms of Action†

Diane D. Ilsley,‡ Suk-Hee Lee,§ Wayne H. Miller,|| and Robert D. Kuchta*,‡

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309, Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101, and Division of Experimental Therapy, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

Received August 25, 1994; Revised Manuscript Received November 7, 1994®

ABSTRACT: Acyclovir triphosphate, ganciclovir triphosphate and penciclovir triphosphate inhibited DNA polymerases α, δ, and ε. Each triphosphate preferentially inhibited pol δ, although ganciclovir triphosphate was the most impressive of the three; the K_i for inhibition of pol δ was 2 μM (competitive with dGTP), while the K_is for inhibition of pol α and ε were 80 and 140 μM, respectively. Each of the compounds was polymerized by pol α, δ, and ε. Incorporation of acyclovir triphosphate resulted in immediate chain termination, whereas incorporation of ganciclovir triphosphate often allowed polymerization of additional dNTPs. Interestingly, chain termination most often occurred after polymerization of just one additional dNTP onto the ganciclovir monophosphate. All three compounds were very weak inhibitors of DNA primase. Acyclovir triphosphate, however, was a unique inhibitor of the pol α-catalyzed elongation of primase-synthesized primers. Immediately after DNA primase synthesized a primer, pol α frequently incorporated acyclovir triphosphate with consequent chain termination. If, however, pol α did not immediately polymerize acyclovir triphosphate onto the primase-synthesized primer, further dNTPs were readily added and acyclovir triphosphate was incorporated much less frequently.

Acyclic guanosine analogs are potent antiviral agents against members of the herpes virus family. Acyclovir and penciclovir (Figure 1) selectively inhibit herpes simplex virus replication (Elion et al., 1977; Vere Hodge, 1993), while ganciclovir potently inhibits cytomegalovirus replication (Field et al., 1983; Freitas et al., 1985). The active metabolites of these compounds are the corresponding triphosphates. Selective inhibition of viral replication by these compounds largely occurs because phosphorylation of the nucleoside to the nucleoside monophosphate is dependent upon a virally-encoded protein (Elion et al., 1977; Fyfe et al., 1978; Biron et al., 1985), with subsequent phosphorylation to the triphosphate catalyzed by cellular kinases (Miller & Miller, 1980, 1982). More potent inhibition of the viral polymerases compared to human polymerase α may also contribute to the selectivity of these compounds (Furman et al., 1980; Derse et al., 1981).

In addition to its antiviral properties, ganciclovir is quite cytotoxic and can have severe side effects due to its toxicity toward bone marrow cells (Faulds & Heel, 1990). The acyclic guanosine analogs are poor substrates for the human nucleoside kinases (Hovi & Field, 1988), but low levels of the triphosphates are none-the-less detected in uninfected cells (Biron et al., 1985; Vere Hodge, 1993). These low levels of ganciclovir triphosphate (GCVTP)[1] are thought to cause the side effects associated with ganciclovir therapy. More recently, an experimental cancer chemotherapy that takes advantage of the activation of ganciclovir by viral kinases and the potent cytoxicity of the resultant GCVTP has been developed (Culver et al., 1992).

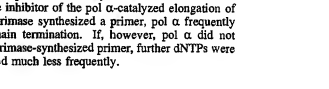


FIGURE 1: Structures of acyclovir, ganciclovir, and penciclovir.

Previous studies have shown that ACVTP, GCVTP, and PCVTP are poor inhibitors of human pol α relative to the HSV DNA polymerase (Frank et al., 1984; Reardon, 1989; Reardon & Spector, 1989; Vere Hodge, 1993). However, these studies only included inhibition of pol α activity, and chromosomal DNA replication likely requires three DNA polymerases, pol α, δ, and ε. In addition, pol α copurifies as a complex with a second activity, DNA primase,[2] such that on single-stranded DNA, primase synthesizes short RNA primers which are then elongated by pol α (Grosse & Krauss,

† This work was supported by grants from the National Science Foundation (DMB-9105808, R.D.K.), the Council for Tobacco Research (2612A, R.D.K.), and the American Lebanese Syrian Associated Charities of Saint Jude Children's Research Hospital (S.-H.L.). R.D.K. was partially supported by a Junior Faculty Research Award from the American Cancer Society (337).

* Address correspondence to this author. Telephone: (303) 492−7027. FAX: (303) 492−5894. EMail: kuchta@spot.colorado.edu.

‡ University of Colorado.

§ Saint Jude Children's Research Hospital.

|| Wellcome Research Laboratories.

® Abstract published in *Advance ACS Abstracts,* February 1, 1995.

[1] Abbreviations: ACVTP, acyclovir triphosphate; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid, sodium salt; GCVTP, ganciclovir triphosphate; HSV, herpes simplex virus; PCNA, proliferating cell nuclear antigen; PCVTP, penciclovir triphosphate; pol α, DNA polymerase δ; pol δ, DNA polymerase δ; pol ε, DNA polymerase ε; Tris, tris(hydroxymethyl)aminomethane, HCl salt.

[2] We shall use the terms "primase" and "pol α" to denote the primase and polymerase components of the pol α−primase complex. Unless noted otherwise, the calf thymus pol α−primase complex was used for all experiments.

0006-2960/95/0434-2504$09.00/0 © 1995 American Chemical Society

Exhibit 5a

Table 1: Synthetic Oligodeoxynucleotides of Defined Sequence

oligodeoxynucleotide	sequence
DNA_G	TCCATATCACAT-3′ AGGTATAGTGTAGATCTTATCATCT
DNA_T	TCCATATCACATGCGCCG-3′ AGGTATAGTGTACGCGGCTTTGTGTAATAGTAAG
DNA_{Chad}	CAAACTGTGATACGATGGACTAGCTGAATC-3′ GTTTGACACTATGCTACCTGATCGACTTAGGAGCCTGCTATCGG
$d(TCC)_{20}$	5′-TCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCC
$d(TTC)_{13}$	5′-TTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTC

1985; Hu et al., 1984). Effects of ACVTP, GCVTP, and PCVTP on these other enzymes have not been examined.

Using synthetic oligodeoxynucleotides of defined sequence, we investigated the effects of ACVTP, GCVTP, and PCVTP on DNA primase and pol α, δ, and ε. GCVTP selectively inhibited pol δ relative to the other replicative enzymes. ACVTP and PCVTP also targeted pol δ, but with decreased selectivity. Furthermore, we found that ACVTP has a unique mechanism for inhibiting pol α activity that only occurs when pol α elongated a primase-synthesized primer.

EXPERIMENTAL PROCEDURES

Materials

Unless noted, all materials were as described previously (Kuchta et al., 1992; Sheaff et al., 1991). Human pol α catalytic subunit was a generous gift from Dr. Bill Copeland at NIEHS, Research Triangle Park, NC, and human pol ε was kindly provided by Dr. Peng Huang and Dr. William Plunkett, M. D. Anderson Hospital, Houston, TX. Human pol δ, calf thymus pol α−primase, and PCNA were purified as described previously (Lee et al., 1989; Huang et al., 1991; Sheaff et al., 1991). A unit of activity is defined as polymerization of 1 nmol of dNTP h^{-1} under the previously described conditions for each enzyme (Lee et al., 1989; Sheaff et al., 1991). Synthetic primer-templates were synthesized and quantified as described previously (Sheaff et al., 1991; Sheaff & Kuchta, 1993). DNA_{Chad} was a generous gift of Chad Greef and Dr. Marvin Caruthers (University of Colorado). The concentrations of synthetic oligodeoxynucleotides are given in terms of 3′-termini.

PCVTP and GCVTP were enzymatically synthesized as described previously (Ertl et al., 1994). ACVTP was synthesized as described by Furman et al. (1979).

Methods

All assays were performed at 37 °C and contained 50 mM Tris, pH 7.5, and 5 mM $MgCl_2$. Reactions were initiated by addition of enzyme, and rates were measured under initial velocity conditions. All reactions were quenched by the addition of either 1.5 volumes of 50 mM EDTA, pH 7.5, or 1 volume of gel loading buffer (90% formamide). Radioactivity incorporated into products was measured either by a DE81 filter binding assay (Sheaff et al., 1991) or by polyacrylamide gel electrophoresis (18% acrylamide/8 M urea; Sheaff et al., 1991) followed by phosphorimagery on a Molecular Dynamics PhosphorImager.

Polymerase Activity. Assays (6−10 μL) contained 1 μM DNA, 5−20 μM dNTPs (with either [α-^{32}P]dATP, -dCTP, or -dTTP at a specific activity of ca. 1×10^5 cpm $pmol^{-1}$), 2 mM DTT, 0.05 mg mL^{-1} BSA, enzyme [1 unit of pol α, 0.4 unit of pol ε, or 0.1 unit of pol δ (16 ng) plus 35 ng of PCNA], and various concentrations of the acyclic analogs. For K_i and IC_{50} measurements, the amount of product was determined using the DE81 filter binding assay.

Primase and Primase-Coupled Pol α Activities. Primase activity was measured in assays (6−10 μL) containing 1 μM template, 50 μM [α-^{32}P]GTP (ca. 20 000 cpm $pmol^{-1}$), 50 μM ATP, 0.1 mg mL^{-1} BSA, 2 units of pol α-primase (measured as pol α activity), and various concentrations of the acyclic analogs. Reactions were quenched with gel loading buffer (90% formamide), and products were analyzed by gel electrophoresis and phosphorimagery. In those primase-coupled pol α activity assays where we wanted to visualize both primase and polymerase products, conditions were identical to primase assays except that they also contained 5 μM dATP and dGTP. Primase-coupled pol α activity assays in which only the polymerase products were visualized contained 1 μM template, 200 μM ATP and GTP, 0.1 mg mL^{-1} BSA, 2.5−20 μM dGTP, 2.5−20 μM [α-^{32}P]-dATP, 2 units of pol α−primase, and various concentrations of the acyclic analogs.

Polymerization of the Acyclic Analogs. Assays (6 μL) contained 1 μM DNA_G, 5 μM [α-^{32}P]dATP, dTTP, and dCTP, 2 mM DTT, 0.05 mg mL^{-1} BSA, enzyme (1 unit of pol α, 0.4 unit of pol ε, or 0.1 unit of pol δ plus 35 ng of PCNA), and either 5 μM dGTP or the indicated concentration of inhibitor. Reactions which contained pol α or δ were quenched after 5 min, while those containing pol ε were quenched after 16 min by the addition of an equal volume of gel loading buffer, and the products were analyzed by gel electrophoresis and phosphorimagery.

Elongation of Primase-Synthesized Primers by Klenow Fragment. Primase assays were performed as described above. Reactions were quenched after 30 min by heating to 65 °C for 5 min to denature pol α−primase, followed by a slow cool to room temperature to allow the primers to reanneal to the templates. $MgCl_2$ (2.5 mM), 625 μM of each dNTP, and 0.1 unit μL^{-1} Klenow fragment (exo⁻) were now added (final concentrations), and the reactions were quenched after 15 min by the addition of 1 volume of gel loading buffer. Products were analyzed by gel electrophoresis and phosphorimagery.

RESULTS

Synthetic DNAs of defined sequence were used as substrates for these studies (e.g. DNA_G, Table 1). DNA_G is a good substrate for both pol α and pol ε (Sheaff et al., 1991; Cheng & Kuchta, 1993), and initial experiments demonstrated that in the presence of PCNA, DNA_G is also a good substrate for pol δ. Whereas pol δ exhibited little activity

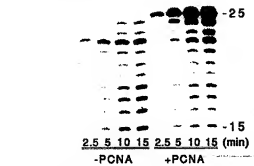


FIGURE 2: DNA_G is a good substrate for pol δ and PCNA. Assays contained 1 μM DNA_G, 6 μM [α-^{32}P]dATP, dCTP, dGTP, and dTTP, 2 mM DTT, 0.1 mg mL^{-1} BSA, and 16 ng of pol δ. At the indicated times, the reactions were stopped and analyzed by gel electrophoresis followed by phosphorimagery. The absence or presence of PCNA (35 ng) and the locations of the 15-mer and 25-mer products are noted on the gel.

Table 2: K_i and IC_{50} Values for the Acyclic Compounds

	K_i (μM)[a]		IC_{50} (μM)[b]		
	GCVTP	ACVTP	GCVTP	ACVTP	PCVTP
pol α	80)	23	100 (125)	30 (90)	450
pol δ	2	2.7	3 (7)	4 (8)	120
pol ϵ	140	50	200 (250)	65 (125)	375

[a] K_i values for each polymerase were measured via Dixon plots using 1 μM DNA_G and varying all four dNTPs simultaneously, as described under Experimental Procedures. Values given are ±20%. Calf thymus pol α was used for these experiments. [b] IC_{50} values were measured in assays that contained either 1 μM DNA_G or 1 μM DNA_{Chol}, and 6 μM dNTPs for pol α and δ, and 5 μM dNTPs for pol ϵ. The values in parentheses are the IC_{50} values when DNA_{Chol} was the substrate.

and very low processivity when PCNA was not present. Figure 2 shows that the addition of PCNA increased both the processivity and the rate of dNTP polymerization (12-fold), consistent with the previously reported effects of PCNA on pol δ (Prelich et al., 1987; Bambara & Jessee, 1991). In the presence of 10 μM dNTPs and PCNA, measuring the rate as a function of DNA_G concentration gave a V_{max} value of 1600 nmol of dNTP (30 min)$^{-1}$ mg^{-1}, and a $K_{M(DNA)}$ value of 4 μM. Thus, even these very short DNAs allow productive pol δ/PCNA interactions and can be used for inhibition studies. Furthermore, since PCNA appears to be an essential cofactor for pol δ, PCNA was included in all further studies of pol δ activity.

Inhibition of Pol α, δ and ϵ by ACVTP, GCVTP and PCVTP. Inhibition of pol α, δ, and ϵ by the acyclic analogs was measured using DNA_G as substrate (Table 2). In each case, inhibition by the compounds was competitive with respect to dGTP (data not shown).[3] Impressively, the K_i for inhibition of pol δ by GCVTP was 40- and 70-fold lower than for pol α and ϵ, respectively. Table 2 also shows the IC_{50} values for inhibition by GCVTP at a single dNTP concentration in order to allow a direct comparison of the relative potency of inhibition under identical conditions. Importantly, since inhibition was measured on the identical template with all three polymerases, differences in the potency of inhibition were not due to differences in the template composition. To ensure that the selective inhibition of pol δ activity by GCVTP was not limited to this particular template, we next examined inhibition of each polymerase using a second DNA that had a different sequence and a longer duplex region (DNA_{Chol}, Table 1). Again, GCVTP preferentially inhibited pol δ (Table 2).

[3] Consistent with GCVTP being a dGTP analog, GCVTP did not inhibit pol δ activity on DNA_T (Table 1), a DNA that lacks any deoxycytidylates in the single-stranded template.

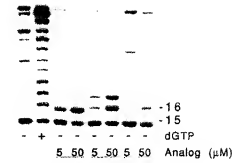


FIGURE 3: Polymerization of the acyclic analogs by pol δ. Assays contained 1 μM DNA_G and 5 μM dCTP, dTTP, and [α-^{32}P]dATP. The presence of 5 μM dGTP and the indicated concentrations of ACVTP (ACV), GCVTP (GCV), and PCVTP (PCV) are as noted. The first marked product (15-mer) represents polymerization of dCTP, dTTP, and [α-^{32}P]dATP onto the primer. The second product (16-mer) results from polymerization of dGTP or the acyclic analog. The small amount of 26-mer synthesized in each assay is probably an "end-addition" product due to polymerization of [α-^{32}P]dATP onto the 3'-end of the template strand.

ACVTP also inhibited pol δ more potently than pol α and ϵ, albeit with less selectivity (Tables 2 and 3). PCVTP, on the other hand, poorly inhibited all three polymerases such that we only determined IC_{50} values rather than K_is. Similar to ACVTP, PCVTP also showed slight specificity toward pol δ inhibition.

Pol α, δ, and ϵ Incorporate ACVTP, GCVTP, and PCVTP into DNA. Pol δ can use ACVTP, GCVTP, and PCVTP as substrates (Figure 3). The first lane shows the products synthesized by pol δ in an assay containing only DNA_G, [α-^{32}P]dATP, dCTP, and dTTP. The primary product that accumulated, a 15-mer, did so because the next required dNTP, dGTP, was omitted from the reaction.[4] If either ACVTP or GCVTP was now included in the assays, new products were synthesized. These new products were primarily 1−2 nucleotides longer than the major product synthesized in the absence of the analog, consistent with incorporation of the analogs. The presence of PCVTP results in the synthesis of at most small amounts of new products. The acyclic analogs appear to be poor substrates for pol δ, since even in the presence of 50 μM ACVTP, GCVTP, or PCVTP, pol δ incorporated the analogs into only a fraction of the DNA−the enzyme frequently dissociated rather than

[4] In the absence of dGTP, pol δ polymerized nucleotides past the template dC approximately 6% of the time. This surprisingly large amount of products longer than the 15-mer might be a function of the template sequence or size, or could be due to trace amounts of dGTP as an impurity in the other dNTPs.

incorporate the analog. When 50 μM ACVTP or 50 μM GCVTP was included in the assay, only 66% and 62%, respectively, of the 15-mer was elongated to a 16-mer (or longer product) via analog incorporation. In contrast, including only 5 μM dGTP resulted in elongation of 90% of the 15-mer into longer products. It should be noted, however, that these may be underestimates of the ability of pol δ to polymerize the analogs since the 3′ → 5′ exonuclease activity of pol δ may have excised some of the analog incorporated by the polymerase activity.

Consistent with ACVTP being a chain-terminator, incorporation of ACVTP inhibited any further polymerization by pol δ (Figure 3). Conversely, incorporation of GCVMP to generate the 16-mer did not necessarily result in chain termination; one additional dNMP was polymerized onto 33% and 28% of the 16-mer (5 or 50 μM GCVTP, respectively). Polymerization of this additional dNTP usually resulted in chain termination, as longer products were rarely observed. These data indicate that pol δ will recognize primers that contain a GCVMP at the 3′-terminus or one nucleotide removed from the 3′-terminus; however, they are utilized relatively inefficiently as substrates. PCVTP could also be polymerized by pol δ, albeit less efficiently. This is consistent with PCVTP being a weaker inhibitor of pol δ than GCVTP and ACVTP. Small amounts of products due to polymerization of multiple dNTPs were synthesized in assays containing GCVTP and PCVTP. This could result from either polymerization of multiple dNTPs onto the GCVMP or PCVMP or misincorporation opposite the template dC followed by polymerization of multiple dNTPs.

Pol α and pol ϵ could also incorporate ACVTP, GCVTP, and PCVTP into primer-templates, and the results were extremely similar to those with pol δ (data not shown). Incorporation of ACVTP resulted in immediate chain termination, whereas incorporation of GCVTP usually resulted in either immediate chain termination or polymerization of one additional dNTP followed by chain termination. PCVTP was also incorporated, albeit less efficiently than ACVTP or GCVTP. Polymerization of multiple dNTPs following incorporation of GCVTP or PCVTP rarely occurred.

Acyclic Analogs Are Poor Inhibitors of Primase Activity. Effects of ACVTP, GCVTP, and PCVTP on primase activity were examined using templates of defined sequence $d(TCC)_{20}$ and $d(TTC)_{13}$, Table 1]. Once primase synthesizes a primer 7−10 nucleotides long (i.e., a unit-length primer), the newly generated primer-template is transferred intramolecularly from primase to pol α, whereupon further primase activity is negatively regulated until pol α elongates this primer-template (Sheaff et al., 1994). This negative regulation of primase activity after synthesis of a single unit-length primer results in a biphasic time-course of primase activity (Sheaff & Kuchta, 1993). Including either GCVTP (Figure 4) or ACVTP (data not shown) in primase assays had no effect on negative regulation, although the rate of primer synthesis decreased.

In order to precisely measure the effect of ACVTP, GCVTP, and PCVTP on primase activity, we examined steady-state rates of primer synthesis. The normal, biphasic time course of primer synthesis can be converted to a linear time course by preventing the newly generated primer-template from interacting with the pol α active site (Sheaff et al., 1994). This can be accomplished by including an

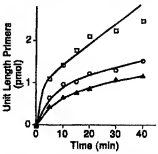


FIGURE 4: Time course of primase activity on $d(TCC)_{20}$ remains biphasic in the presence of GCVTP. Reactions contained 1 μM $d(TCC)_{20}$, 50 μM ATP, 50 μM [α-^{32}P]GTP, and 0 (□), 100 (○), or 180 μM (▲) GCVTP, and were performed as described under Experimental Procedures.

exogenously added primer-template (DNA_G) and aphidicolin in the assays. Under these conditions, a pol α-DNA_G-aphidicolin ternary complex forms and prevents primase-generated primer-templates from interacting with the pol α active site. This alleviates the negative regulation of primase activity that normally occurs after synthesis of a unit-length primer but does not otherwise affect primase activity (Sheaff et al., 1992, 1994). Under these steady-state conditions, ACVTP, GCVTP, and PCVTP weakly inhibited primase activity in assays containing $d(TCC)_{20}$ and 50 μM [α-^{32}P]-NTPs (IC_{50} = 100, 250, and 190 μM, respectively).

Pol α Incorporates ACVTP, GCVTP and PCVTP onto Primase Synthesized Primers. Figure 5 shows the effects of ACVTP, GCVTP and PCVTP on the products synthesized by DNA primase. The altered mobility of the longer primers suggested that the analogs were polymerized onto the primers. Klenow fragment was used to explicitly show that primers synthesized in the presence of ACVTP contained ACVMP at the 3′-terminus. Klenow fragment readily elongated primase-synthesized primers synthesized in the absence of ACVTP (Figure 5). However, if ACVTP was included during primer synthesis, Klenow fragment could no longer elongate the primers, indicating that the primers contained ACVMP at the 3′-terminus. Control experiments showed that the ACVTP did not inhibit Klenow fragment.[5]

To show that pol α was responsible for polymerizing the ACVTP, GCVTP, and PCVTP onto the primase-synthesized primers, aphidicolin and DNA_G were included in the assays in order to inhibit pol α (Figure 5). As expected, inhibition of pol α did not affect the pattern of products when only ATP and GTP were present. In contrast, inhibition of pol α eliminated the synthesis of products with altered mobility when the reactions contained either ACVTP, PCVTP, or GCVTP, and restored the pattern of products to that observed when only ATP and GTP were present. Thus, pol α polymerized the analogs onto the primase-synthesized primers.

Inhibition of Primase-Coupled Pol α Activity. On a single-stranded DNA, primase synthesizes primers that can be immediately elongated by pol α (i.e., primase-coupled pol

[5] Klenow fragment will slowly elongate primers containing a 3′-terminal GCVMP (Reardon, 1989) or a 3′-terminal PCVMP (data not shown). The primers of altered mobility that were synthesized in assays containing GCVTP or PCVTP were elongated by Klenow fragment more slowly than "normal" primers, suggesting that they contained GCVMP or PCVMP, respectively, at the 3′-terminus.

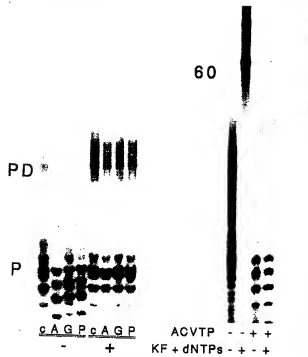


FIGURE 5: Pol α polymerizes ACVTP, GCVTP, and PCVTP onto primase-synthesized primers. Lanes marked c show the products from assays containing 1 μM d(TCC)$_{20}$, 0.1 mg mL^{-1} BSA, 50 μM ATP, and 50 μM [α-^{32}P]GTP. The presence of either 50 μM ACVTP (A), 50 μM GCVTP (G), or 50 μM PCVTP (P) in the assays is noted. Additionally, the assays marked with a "+" also contained 5 μM DNA$_G$ and 50 μM aphidicolin to inhibit pol α, while the assays marked "−" lacked these compounds. The higher molecular weight products labeled PD are primer-dimers (Sheaff & Kuchta, 1993), products that are only formed in the absence of dNTPs. Primers synthesized in the absence (−) or presence (+) of ACVTP were then treated with Klenow fragment (KF) and dNTPs as described under Experimental Procedures. The location of a 60-nucleotide-long standard is shown.

Table 3: Inhibition of Primase-Coupled Pol α Activity by Acyclic Nucleotide Analogs[a]

analog	K_i (μM) for substrate	
	d(TCC)$_{20}$	d(TTC)$_{13}$
ACVTP	1	8
GCVTP	15	60
PCVTP	200	nd[b]

[a] Reactions contained 1 μM template, 200 μM GTP and ATP, 2.5–20 μM [α-^{32}P]dATP and dGTP, and varying concentrations of inhibitor. Apparent K_i values were determined via Dixon plots at three dNTP concentrations. [b] Not determined.

α activity). Inhibition of primase-coupled pol α activity by ACVTP, GCVTP, and PCVTP was measured in assays containing d(TCC)$_{20}$, NTPs, dGTP, and [α-^{32}P]dATP. Table 3 shows that each acyclic nucleotide inhibited [α-^{32}P]dATP polymerization. Inhibition was competitive with respect to dGTP, indicating that the reduced rate of [α-^{32}P]dNTP polymerization was due to inhibition of pol α. The more potent inhibition of primase-coupled pol α activity on d(TCC)$_{20}$ than on DNA$_G$ is likely due to the higher percentage of template dC's in d(TCC)$_{20}$. Consistent with this idea, inhibition of primase-coupled pol α activity on the template d(TTC)$_{13}$ was substantially less potent than inhibition on d(TCC)$_{20}$ (Table 3).

FIGURE 6: Inhibition of primase-coupled pol α activity by ACVTP, PCVTP, and GCVTP. Assays contained pol α–primase, 1 μM d(TCC)$_{20}$, 50 μM ATP, 50 μM [α-^{32}P]GTP, 5 μM dATP, 5 μM dGTP, and the indicated concentrations of ACVTP, PCVTP, or GCVTP. Lane c contained no inhibitor. The length of oligonucleotide standards is shown.

We then analyzed the effects of the acyclic nucleotides on the size distribution of products synthesized during the pol α-catalyzed elongation of primase-synthesized primers. Assays contained d(TCC)$_{20}$, [α-^{32}P]GTP, ATP, dGTP, and dATP; thus, all products would be labeled. In the absence of any inhibitor, pol α efficiently elongated the primase-synthesized primers into products 50–60 nucleotides long (Figure 6). GCVTP decreased the total amount of elongated products; however, there were no significant changes in the sizes of products. In contrast, ACVTP decreased the total amount and length of elongated product. Interestingly, large amounts of products ca. 10 nucleotides long accumulated at even low concentrations of ACVTP, but the amount of intermediate length products between ca. 12 and 45 nucleotides long increased much less. For example, addition of 20 μM ACVTP increased the amount of products ca. 10 nucleotides long by 130%, whereas the amounts of products from 12 to 45 nucleotides long increased by only 30% and products greater than 45 nucleotides long decreased by 65%. Addition of PCVTP affected primase-coupled pol α activity similarly to ACVTP, in that it also resulted in the accumulation of products ca. 10 nucleotides long.

Accumulation of products ca. 10 nucleotides long in the presence of ACVTP suggested that pol α had polymerized

ACVTP onto the primase-synthesized primers. This was examined using Klenow fragment as described earlier. Klenow fragment could not elongate the products ca. 10 nucleotides long that accumulated in the presence of ACVTP and dNTPs, whereas Klenow fragment readily elongated primers synthesized in the absence of ACVTP. Thus, these products ca. 10 nucleotides long contain ACVMP at the 3′-terminus.

The relative efficiency for polymerization of dGTP versus ACVTP during elongation of primase-synthesized primers was determined. Polymerization of ACVTP onto a primase-synthesized primer results in a product ca. 10 nucleotides long, whereas polymerization of dGTP results in products >10 nucleotides long. Thus, the relative frequency for dGTP versus ACVTP polymerization can be quantified as a function of [dGTP]/[ACVTP] in order to calculate $(k_{cat}/K_M)_{dGTP}/(k_{cat}/K_M)_{ACVTP}$ (Kuchta et al., 1992). During elongation of primase-synthesized primers, pol α preferred to polymerize dGTP rather than ACVTP by only a factor of 5.

DISCUSSION

The acyclic nucleotides ACVTP, GCVTP, and PCVTP inhibit DNA pol α, δ, and ε. All three compounds inhibit pol δ more potently than either α or ε, although GCVTP is the most impressive among them. A major question in DNA replication and repair is the respective roles of pol δ and ε, since both polymerases share several common features including accessory proteins and a 3′ → 5′ exonuclease. The ability of GCVTP to discriminate between pol δ and ε may be very useful for elucidating the roles of pol δ and ε. Additionally, the potent inhibition of pol δ by GCVTP suggests that inhibition of cellular DNA replication induced by treatment with ganciclovir is likely due to inhibition of pol δ.

Each DNA polymerase could incorporate all three compounds into DNA. Unlike ACVTP, however, GCVTP and PCVTP are not absolute chain terminators since they contain the equivalent of a 3′-hydroxyl. Interestingly, chain termination primarily occurred immediately after the polymerases had incorporated GCVTP as well as after polymerization of one additional dNTP onto the GCVMP. Reardon (1989) reported that pol α elongates primers containing a GCVMP either at the primer 3′-terminus or one nucleotide away from the primer 3′-terminus very inefficiently compared to normal primers containing only dNMPs, consistent with the chain termination we observed. HSV DNA polymerase has also been reported to incorporate GCVMP and one additional dNTP into DNA, but then have great difficulty in polymerizing further dNTPs (Reid et al., 1988). This phenomenon is not limited to acyclic nucleotides, since pol α and ε will polymerize 2′,2′-difluoro-2′-deoxycytidine triphosphate followed by one additional dNTP, and then terminate further dNTP polymerization (Huang et al., 1991). It should be noted, however, that termination after incorporation of a nucleotide analog followed by one additional dNTP is not a general result of 2′-modification of nucleotides, since araNTPs tend to be immediate chain terminators of DNA synthesis by purified polymerases (Huang et al., 1991; Townsend & Cheng, 1987).

The ability of DNA polymerases to incorporate GCVTP, and perhaps also PCVTP, and then polymerize additional dNTPs may have important *in vivo* consequences. PCVTP and GCVTP, both of which are mutagenic [Physicians Desk Reference (1994); SmithKline (1994)], could be incorporated into internucleotide linkages of cellular DNA during DNA replication or repair with consequent mutagenic and/or cytotoxic effects during the next S-phase when the cell attempts to replicate past the analog. Significantly, the effects of acyclovir, ganciclovir, and penciclovir on cellular DNA replication will be most severe in cells infected with a herpes virus. Whereas infected cells accumulate high levels of each triphosphate, uninfected cells accumulate only low levels.

Inhibition of Primase-Coupled Pol α Activity by ACVTP. ACVTP was a moderate inhibitor of pol α activity when an exogenously added primer-template was the substrate, consistent with previous studies on pol α inhibition (Reardon, 1989; Vere Hodge, 1993). However, ACVTP more potently inhibited primase-coupled pol α activity than pol α activity with exogenously added DNA primer-templates, and pol α readily polymerized ACVTP onto primase-synthesized primers. The facile polymerization of ACVTP onto primase-synthesized primers and consequent chain termination may cause the more potent inhibition of primase-coupled pol α activity compared to inhibition of dNTP polymerization onto an exogenously added primer-template. The relative paucity of products that are of intermediate length between fully elongated primase-synthesized primers (ca. 50−60 nucleotides long) and primase-synthesized primers containing an ACVMP at the 3′-terminus (ca. 10 nucleotides long) indicates that pol α either immediately polymerizes ACVTP onto the primase-synthesized primer or, failing this, polymerizes ACVTP into products much less readily. Consistent with this idea, pol α polymerizes dGTP 100-fold more readily than it polymerizes ACVTP when an exogenously added DNA primer-template is used as the substrate [V_{max}/K_M (Reardon, 1989)]. This may be equivalent to the situation if pol α fails to polymerize ACVTP onto the primase-synthesized primer. In contrast, pol α prefers to polymerize dGTP rather than ACVTP by a factor of 5 (V_{max}/K_M) during polymerization of the first nucleotide(s) onto the primase-synthesized primer. Interestingly, PCVTP appears similar to ACVTP regarding polymerization onto primase-synthesized primers and consequent accumulation of products ca. 10 nucleotides long (Figure 6), whereas GCVTP does not have this effect.

This reduced ability of pol α to discriminate against ACVTP could either be a property of polymerization onto an RNA primer or be a unique feature of primase-coupled pol α activity. We previously found that changing the primer from DNA to RNA could dramatically alter how pol α interacts with nucleotide analogs (Kuchta et al., 1992). For example, pol α discriminated against 2′,3′-dideoxy-NTP polymerization 85-fold less efficiently when the primer was RNA instead of DNA. More recently, we have found that primase-coupled pol α activity also has unique properties regarding the ability of araNTPs to act as chain terminators (H. Thompson and R. Kuchta, unpublished data).

Importantly, these data suggest that the addition of the first nucleotide(s) onto a primase-synthesized primer may be uniquely sensitive to inhibition by nucleotide analogs, and kinetic parameters for polymerization of these first few nucleotide(s) may be very different than the parameters for polymerization of nucleotides onto exogenously added primer-templates. Furthermore, when considering the effects

of a nucleotide analog on DNA replication, it may be more accurate to study primase-coupled pol α activity, as opposed to pol α activity on an exogenously added primer-template. Whereas pol α is almost certainly coupled to primase activity during DNA replication, it is unclear if pol α ever uses a "pre-existent" DNA primer-template during DNA replication.

ACKNOWLEDGMENT

We thank Carol J. Bixler and Matthew P. Barrett for assistance during the synthesis of PCVTP.

REFERENCES

Bambara, R. A., & Jesse, C. B. (1991) *Biochim. Biophys. Acta 1088*, 11−24.

Biron, K. K., Stanat, S. C., Sorrell, T. B., Fyfe, J. A., Keller, P. M., Lambe, C. U., & Nelson, D. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2473−2477.

Cheng, C.-H., & Kuchta, R. D. (1993) *Biochemistry 32*, 8568−8574.

Culver, K. W., Ram, Z., Walbridge, S., Ishii, H., Oldfield, E. J., & Blaese, R. M. (1992) *Science 256*, 1150−1152.

Derse, D., Cheng, Y.-C., Furman, P. A., St. Clair, M. H., & Elion, G. B. (1981) *J. Biol. Chem. 256*, 11447−11451.

Elion, G. B., Furman, P. A., Fyfe, J. A., DeMiranda, P., Beauchamp, L., & Schaeffer, H. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5716−5720.

Ertl, P., Snowden, W., Walters, A., Gower, D., Miller, W. H., Collins, P., & Littler, E. (1994) *Antiviral Chem. Chemother.* (in press).

Faulds, D., & Heel, R. C. (1990) *Drugs 39*, 597−638.

Field, A. K., Davies, M. E., DeWitt, C., Perry, H. C., Liou, R., Germershausen, J., Karkas, D., Ashton, W. T., Johnston, D. B. R., & Tolman, R. L. (1983) *Proc. Natl. Acad. Sci. U.S.A.* 80, 4139−4143.

Frank, K. B., Chiou, J.-F., & Cheng, Y.-C. (1984) *J. Biol. Chem. 259*, 1566−1569.

Freitas, V. R., Smee, D. F., Chernow, M., Boehme, R., & Matthews, T. R. (1985) *Antimicrob. Agents Chemother. 28*, 240−245.

Furman, P. A., St. Clair, M. H., Fyfe, J. A., Rideout, J. L., Keller, P. M., & Elion, G. B. (1979) *J. Virol. 32*, 72−77.

Furman, P. A., McGuirt, P. V., Keller, P. M., Fyfe, J. A., & Elion, G. B. (1980) *Virology 102*, 420−430.

Fyfe, J. A., Keller, P. M., Furman, P. A., Muller, R. L., & Elion, G. B. (1978) *J. Biol. Chem. 253*, 8721−8727.

Grosse, F., & Krauss, G. (1985) *J. Biol. Chem. 260*, 1881−1888.

Hovi, T., & Field, H. J. (1988) *Antiviral Agents: The Development and Assessment of Antiviral Chemotherapy*, Vol. 1, CRC Press, Boca Raton, FL.

Hu, S.-Z., Wang, T. S. F., & Korn, D. (1984) *J. Biol. Chem. 259*, 2602−2609.

Huang, P., Chubb, S., Hertel, L. W., Grindey, G. B., & Plunkett, W. (1991) *Cancer Res. 51*, 6110−6117.

Kuchta, R. D., Ilsley, D., Kravig, K. D., Schubert, S., & Harris, B. (1992) *Biochemistry 31*, 4720−4728.

Lee, S.-H., Eki, T., & Hurwitz, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7361−7365.

Miller, W. H., & Miller, R. L. (1980) *J. Biol. Chem. 255*, 7204−7207.

Miller, W. H., & Miller, R. L. (1982) *Biochem. Pharmacol. 31*, 3879−3884.

Physicians Desk Reference (1994) *Cytovene*, pp 2397−2401, Medical Economics Co., Oradell, NJ.

Prelich, G., Tan, C.-K., Kostura, M., Mathews, M. B., So, A. G., Downey, K. M., & Stillman, B. (1987) *Nature 326*, 517−521.

Reardon, J. E. (1989) *J. Biol. Chem. 264*, 19039−19044.

Reardon, J. E., & Spector, T. (1989) *J. Biol. Chem. 264*, 7405−7411.

Reid, R., Mar, E.-C., Huang, E.-S., & Topal, M. D. (1988) *J. Biol. Chem. 263*, 3898−3904.

Sheaff, R., & Kuchta, R. D. (1993) *Biochemistry 32*, 3027−3037.

Sheaff, R., Ilsley, D., & Kuchta, R. (1991) *Biochemistry 30*, 8590−8597.

Sheaff, R., Kuchta, R. D., & Ilsley, D. (1994) *Biochemistry 33*, 2247−2254.

SmithKline Beecham (1994) Package Insert for Famvir (Penciclovir).

Townsend, A. J., & Cheng, Y.-C. (1987) *Mol. Pharmacol. 32*, 330−339.

Vere Hodge, R. A. (1993) *Antiviral Chem. Chemother. 4*, 67−84.

BI941988A

Volume 17 Number 19 1989 Nucleic Acids Research

Conformational aspects of antiviral activity of deoxyguanosine acyclic analogues

A.A.Golbraikh, J.Betins, J.Balodis, R.A.Zhuk and G.V.Nikiforovich

Institute of Organic Synthesis, Latvian SSR Academy of Sciences, Riga 226006, USSR

Received March 2, 1989; Revised and Accepted September 4, 1989

ABSTRACT

Conformational possibilities of a series of deoxyguanosine analogues possessing or lacking antiviral activity were evaluated using methods of the molecular mechanics. Comparison of the spatial structures of acyclic analogues with one another and with the spatial structures of deoxyguanosine demonstrates restricted conformational mobility for compounds devoid of activity. The level of sterically allowed superposition of functional groups from the acyclic moieties of analogues and the corresponding atomic centres of deoxyribose could serve as a criterion of activity. The superposition could be performed in two different ways through either of the nonhydrogen substituents at the C1' atom in the five-membered ring.

INTRODUCTION

Acycloguanosine (acyclovir) is a highly effective selectively acting drug supressing Herpes Simplex Viruses of the types I and II. The selectivity of acyclovir is explained by the fact that it serves as a good substrate for thymidine kinase from virus-infected cells. (Hence this enzyme recognizes acyclovir as a thymidine analogue). The acycloguanosine monophosphate thus formed undergoes phosphorylation by guanylate kinase to diophosphate and further to triphosphate. Therefore, acycloguanosine monophosphate is recognized by guanylate kinase as a guanosine monophosphate analogue. Acycloguanosine triphosphate also acts as inhibitor of DNA-polymerase from virus-infected cells and competes with dGTP as a substrate. In this reaction it is recognized as a dGTP analogue.

The molecular mechanism described above provides a theoretical background for attempts of effective synthesis of acyclic nucleoside analogues. So far, as a rule, only similarity at the level of structural formulas or certain intramolecular atom-atom distan-

EXHIBIT 5b

ces has been taken into account (see ref. [1]). For instance, it has been suggested in ref. [2], that the similarity of C1'-C4' distances in acyclovir and its analogues can serve as a measure of antiviral activity. The next step in this direction could be a comparison of the spatial structures of nucleosides and their acyclic analogues, a major factor affecting the manifestation of biological activities; hence this would involve investigations into conformation-function relationships. We have attempted to apply this approach to deoxyguanosine (compound I in Fig. 1) and its acyclic analogues described in Table 1. It is important to note that the conformational possibilities of natural ribo- and deoxyribonucleosides are fairly similar [3-6]. In our case, however, the task is complicated by the high conformational mobility of acyclonucleosides, making the results of X-ray analysis untenable (see, e.g., ref. [7]). In this connection, methods of molecular mechanics are very useful, whose application to the problem outlined above forms the subject of the present communication.

METHODS

Energy calculations. Theoretical conformational analysis was performed assuming a rigid valence geometry (the values of bond

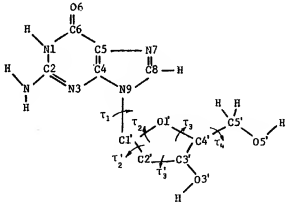
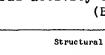
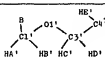
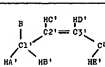
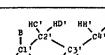
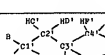
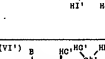
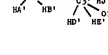


Fig.1. Deoxyguanosine (τ_1(C4-N9-C1'-O1') and τ'_1 (C4-N9-C1'-C2') angles). For the C(2')-endo conformation of deoxyribose $\tau'_1 = \tau_1 - 113^\circ$, for the C(3')-endo conformation of deoxyribose $\tau'_1 = \tau_1 - 118^\circ$.

Table 1. Structural formulas, internal rotation angles and antiviral activity of acyclic analogues of deoxyguanosine (B stands for guanine)

Compound	Structural formula	Angle designations	Activity, LD_{50}, μM
1	2	3	4
(II) (Acyclovir)		τ_1 C4'-N9-C1'-O1' τ_2 N9-C1'-O1'-C3' τ_3 C1'-O1'-C3'-C4' τ_4 O1'-C3'-C4'-O5' τ_5 C3'-C4'-O5'-H5'	0,1 [1]
(III)		τ_1 C4-N9-C1'-C2' τ_2 N9-C1'-C2'-C3' τ_3 C1'-C2'-C3'-C4' τ_4 C2'-C3'-C4'-O5' τ_5 C3'-C4'-O5'-H5'	0.6 [13]
(IV)		τ_1 C4-N9-C1'-C2' τ_2 N9-C1'-C2'-C3' τ_3 O1'-C2'-O3'-C4' τ_4 C2'-C3'-C4'-O5' τ_5 C3'-C4'-O5'-H5'	2.3 [14]
(V)		τ_1 C4-N9-C1'-C2' τ_2 N9-C1'-C2'-C3' τ_3 C1'-C2'-C3'-HE' τ_4 C2'-C3'-C4'-O1' τ_5 C3'-C4'-O1'-HH' τ_6 C2'-C3'-O5'-O2' τ_7 C3'-O5'-O2'-HK'	0.3 [13]
(VI) and (VI')		τ_1 C4-N9-C1'-C2' τ_2 N9-C3-C2'-C3' τ_3 C1'-C2'-C3'-O1' τ_4 C2'-C3'-O1'-HF' τ_5 C1'-C2'-C4'-C5' τ_6 C2'-C4'-O5'-O2' τ_7 C4'-O5'-O2'-HK'	0.2 [14]
(VII) and (VII')		τ_1 C4-N9-C1'-O' τ_2 N9-C1'-O'-C3' τ_3 C1'-O'-C3'-HA' τ_4 N9-C1'-O1'-C3' τ_5 C1'-O1'-C3'-HA' τ_6 O'-C3'-C4'-O1' τ_7 C3'-C4'-O1'-HD' τ_8 O'-C3'-C5'-O2' τ_9 C3'-C5'-O2'-HG'	>250 [14]
(VIII) and (VIII')		τ_1 C4-N9-C1'-O' τ_2 N9-C1'-O'-H2' τ_3 N9-C1'-O'-C3' τ_4 C1'-O'-C3'-C4' τ_5 O'-C3'-C4'-O5' τ_6 C3'-C4'-O5'-H5'	>250 [14]

lengths and valence angles for the base were borrowed from [8], for substituents at N9 from [7] and from data on similar compounds). Atom-atom potential functions, partial charges on atoms and torsional potentials were the same as in [9]. The value of dielectric constant $\varepsilon=2.0$ was used for calculations of electrostatic interactions.

Estimation of variation range for interatomic distances. The largest and smallest values of interatomic distances r_{ij} depending on the set of torsional angles τ_k were determined in the following way: a multidimensional parallelepiped with the edge $\Delta\tau_k$ was randomly filled with multidimensional "points" $\{\tau_k\}$ in accordance with the algorithm described in ref. [10]. The hard sphere potential was used in order to account for the interactions between the atoms involved in distance calculations. Interactions between other atoms were not taken into account. The points $\{\tau_k\}$ causing an overlap of interacting atoms were discarded from the sample.

Estimation of spatial similarity for a pair of conformations. We used the following criterion of spatial similarity for a pair of conformations A and B:

$$D=(1/N)\sum_{i=1}^{N}[(X_i^A - X_i^B)^2 + (Y_i^A - Y_i^B)^2 + (Z_i^A - Z_i^B)^2]$$

where N is the number of atom pairs for superposition and X, Y and Z are the Cartesian coordinates. Conformations were superposed in accordance with the best molecular fit algorithm [11]. We also used a specially elaborated procedure which allows to vary internal coordinates (torsional angles) for one of the conformations while the coordinates of the other remain fixed (deoxyguanosine was considered as a "fixed" molecule in this work). Such changes were restricted by the condition that differences in conformational energy between the global energy minimum and the final point of variations ("energy gap") must not exceed the ΔU value. Thus the flexible molecules of acyclic analogues were "imposed" onto the rigid conformation of the nucleoside.

RESULTS

Insight into the spatial structures of nucleosides (the conformational similarity of different nucleosides was mentioned

Table 2. Range of variation of interatomic distances (Å) in deoxyguanosine (the minimal and maximal distances are given above and below the bar, respectively).

Sugar conformation	Sugar atoms	Base atoms				
		N1	N3	N7	N9	O6
C(2')-endo	O3'	7.2 / 8.0	4.7 / 5.7	6.3 / 6.7	4.3 / 4.6	8.6 / 9.0
	O5'	4.7 / 9.8	2.6 / 7.7	5.0 / 8.0	4.0 / 5.9	6.7 / 10.3
C(3')-endo	O3'	5.3 / 8.6	3.3 / 6.7	5.6 / 6.5	4.4 / 4.5	7.4 / 8.8
	O5'	4.4 / 9.7	2.6 / 7.8	4.8 / 7.9	4.2 / 5.8	6.5 / 10.2

above) was provided by the results of theoretical conformational analysis gained for ribose [5] and adenosine [6]. Thereby sterically allowed regions for the angle τ_1 were selected. The values of τ_2 and τ_3 angles corresponding to the equilibrium conformation of the C(2')-endo and C(3')-endo types for ribose were also obtained in ref. [5] at ε=3.5. The minimal and maximal distances between the atoms which are most important for the manifestation of biological effects were obtained by assuming freely rotating angles τ_1, τ_4, τ_5 and τ_7. These "key" atoms were O3' and O5' in ribose and N1, N3, N7, N9, O6 in the base. The calculations were performed in accordance with the procedure described above. The results are summarized in Table 2.

Energy calculations for the acyclic analogues of the nucleoside described in Table 1 were performed for molecules possessing and lacking antiviral activity (molecules II-VI and VII-VIII, respectively). Since the molecules VI-VIII can exist in two enantiomeric forms and their biological testing was performed for a racemic mixture, each of the forms was treated separately. In total energy calculations were carried out for ten compounds.

The first step of energy calculations included molecules II-V. Conformational energies were minimized for structures representing all combinations of the rotamers t, g^+ and g^- for all angles of internal rotation around single bonds, except for the C-OH bond for which the energetically optimal rotamers were ob-

Table 3. Comparison of one of the calculated conformations for compound II (ΔU=0.92 kcal/mol) with X-ray analysis data [7] (internal rotation angles in degrees).

Angle	X-ray analysis data	One of calculated conformations
τ_1	-76.5	-68
τ_2	-76.0	-70
τ_3	173.2	180
τ_8	60.6	57

tained using the algorithm outlined in ref. [12]. As a result, the lowest conformational energy estimates were obtained for each molecule. The high conformational mobility inferred for the biologically active compounds was based on the fact that 97% of structures for molecule II, 100% for molecules III and IV, and 66% for molecule V are within the 5 kcal/mol energy span. This conclusion is in a good agreement with the results of calculations performed for acyclovir [16].

It is noteworthy that one of the low-energy structures of compound II is fairly similar to that obtained from X-ray analysis data [7] (see Table 3). This fact supports the reliability of calculation methodology used in the present work.

A better insight into the conformational mobility of biologically active molecules could be obtained from conformational maps over the dihedral angles τ_1 and τ_2 for these compounds (Fig. 2). Conformational energy at each point of the map was minimized with respect to all the remaining angles. It should be noted that these obtained maps are fairly similar to those obtained by optimizing the remaining angles in accordance with the algorithm in ref.[12]. Maps for compounds VII and VIII calculated in such a way are presented in Fig. 3. Energy profiles over each τ_3-τ_8 angle were also obtained for the compounds II-VI (the values of other angles were optimized). Thereby the sterically allowed regions for the τ_3-τ_8 angles were determined.

By comparing the maps in Fig. 2 and 3 one can see that the common sterically allowed regions are different for the active (II-VI) and nonactive (VII-VIII) compounds. This fact supports the assumption that conformational factors are important for the

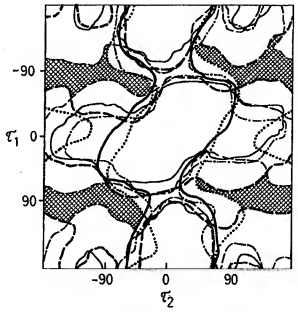


Fig.2. Conformational maps at τ_1 and τ_2 angles for compounds endowed with antiviral activity. The limits of energetically allowed regions ($\Delta U \sim 5$ kcal/mol) for compound II are depicted with thick solid line, for compound III with thin solid line, for compound IV with broken line, for compound V with dot-and-dash line, for compound (VI') with thick broken line. The common area shared by all the maps is dashed.

manifestation of antiviral activity in the given series of compounds. For instance, it can be concluded that the conformational mobility of biologically inactive compounds is restricted as compared with the active ones. However, the regions mentioned above have overlapping parts too. This means that one cannot select "biologically active" conformations for acyclonucleosides in terms of τ_1 and τ_2 values. Moreover, the estimation of variation ranges for intramolecular distances (see Table 4) between the oxygen atom in the acyclic parts of the molecules and the atoms of the base listed in Table 2 demonstrates that the use of these parameters does not allow to distinguish between active and inactive compounds. For all compounds the distance limits in Table 4, which were calculated bearing in mind the previously determined sterically allowed regions $\Delta\tau_k$ for all torsional angles

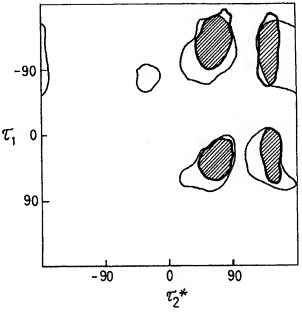


Fig.3. Conformational maps at τ_1 and τ_2 angles for compounds devoid of antiviral activity. (The angles corresponding to τ_2^* angle in acyclovir are τ_4 angle for compound VII and τ_3 angle for compound VIII). The limits of energetically allowed regions ($\Delta U \sim 5$ kcal/mol) for compound VII are depicted with thick line, for compound VIII with thin line.

overlap with the corresponding distance limits from Table 2.

In other words, the hypothesis that the biological activity of compounds is mainly determined by the spatial location of the "key" oxygen atom relative to the base (e.g., [2]) cannot be supported. Apparently the spatial location of other atoms in the acyclic parts of the molecules is also important for mimicking the positions of the corresponding deoxyribose atoms in the nucleoside.

The acyclic parts of compounds from Table 1 could, in principle, be superposed on deoxyribose atoms in two different ways: by going either through atom O1' or through atom C2'. In one of these cases the deoxyribose angles τ_2 and τ_3 could be fixed in conformations corresponding either to the C(2')-endo or C(3')-endo structures, while in the other case the same was valid for the angles τ_2' and τ_3'. The assessment of similarity between flexible acyclic analogues and the nucleoside by using the previously described al-

Table 4. Range of variation of interatomic distances (Å) in acyclic analogues of deoxyguanosine

Compound	Atoms	Base atoms				
		N1	N3	N7	N9	O6
(II)	O5'	2.8 9.9	2.6 7.8	2.6 8.2	2.6 6.0	3.9 10.8
(III)	O5'	2.7 9.4	2.6 7.3	2.6 8.0	2.6 5.8	4.5 10.3
(IV)	O5'	2.6 10.0	2.6 7.9	2.6 8.4	2.6 6.3	4.1 10.9
(V)	O1'	2.6 10.0	2.6 7.9	2.6 8.4	2.6 6.3	4.4 10.8
(V)	O2'	2.6 10.0	2.6 8.0	2.6 8.5	2.6 6.3	4.5 10.9
(VI), (VI')	O2'	2.9 10.0	2.6 8.0	2.6 8.4	2.6 6.3	4.1 10.8
(VII),(VII')	O1'	5.3 9.7	3.1 7.5	5.2 8.2	3.8 6.0	7.0 10.6
(VII),(VII')	O2'	2.6 9.5	2.6 7.4	2.8 8.0	2.6 5.3	4.6 10.2
(VIII), (VIII')	O5'	2.6 9.8	2.6 7.6	2.6 8.3	2.6 6.1	4.4 10.6

gorithm is documented in Table 5. A rigid conformation of the nucleoside with τ_1=-90° was used. The atoms N1, N3, N7, N9, O6 from the base and the atom pairs from deoxyribose and the acyclic analogues mentioned in Table 6 were used for superposition. The "starting" conformations for the flexible acyclic chains were chosed from the sets of τ_1, τ_2, τ_3 angles corresponding to local energy minima close to those in the nucleoside. An energy gap (ΔU) of 6 kcal/mol above the global conformational energy minimum was set for each active compound II-IV. In the case of inactive compound VII and VIII this energy gap was of 8 kcal/mol. The values of conformational energies U corresponding to the global minimum were obtained for every compound on the basis of conformational analysis or conformational maps as described above. Only the results providing the D values less than 1 $Å^2$ are included in Table 5. Two kinds of results are listed in Table 5 for inactive compounds VII and VIII. The results taking into account the position of C2' atom are given in brackets.

The results obtained demonstrate that for all active compounds an excellent spatial superposition ($D \leq 0.1$ $Å^2$) can be achieved within the energy gap $\Delta U \approx 6.0$ kcal/mol. In principle, one

Table 5. Similarity assessment for the molecular geometry of deoxyguanosine and its cyclic analogues (results of superposition with regard to substituent at C1' are given in brackets)

Compound	Deoxyribose conformation	Chain superposed	D, Å^2	ΔU, kcal/mol
(II)	C(2')-endo	O1'	0.00	4.6
	C(3')-endo	O1'	0.00	5.0
(III)	C(2')-endo	O1'	0.41	4.9
	C(2')-endo	C2'-C3'	0.19	5.5
	C(3')-endo	O1'	0.25	3.8
	C(3')-endo	C2'-C3'	0.09	4.0
(IV)	C(2')-endo	O1'	0.05	5.9
	C(3')-endo	O1'	0.02	5.5
(V)	C(2')-endo	O1'	0.04	5.9
	C(3')-endo	O1'	0.16	5.7
(VI)	C(2')-endo	O1'	0.03	5.8
	C(2')-endo	C2'-C3'	0.40	5.9
	C(3')-endo	O1'	0.02	5.2
	C(3')-endo	C2'-C3'	0.41	5.8
(VI')	C(2')-endo	O1'	0.08	6.0
	C(2')-endo	C2'-C3'	0.21	4.8
	C(3')-endo	O1'	0.09	5.3
	C(3')-endo	C2'-C3'	0.32	5.8
(VII)	C(2')-endo	O1'	0.28(0.26)	5.9(5.9)
	C(3')-endo	O1'	0.64(0.68)	5.9(5.9)
(VII')	C(2')-endo	O1'	0.04(0.27)	8.0(8.0)
	C(3')-endo	O1'	0.52(0.89)	5.7(5.7)
(VIII)	C(2')-endo	O1'	0.16(0.25)	5.6(4.9)
	C(3')-endo	O1'	0.24(0.26)	5.9(6.0)
(VIII')	C(2')-endo	O1'	0.05(0.27)	6.9(6.9)
	C(3')-endo	O1'	0.20(0.34)	5.8(6.0)

Table 6. Atoms of acyclic analogues superimposed on deoxyribose atoms

Compound	Deoxyribose atoms					
	C1' C1'	O1' C2'	C4' C3'	C5' C4'	O5' O5'	C2'* - **
(II)	C1'	O1'	C3'	C4'	O5'	-
(III)	C1'	C2'	C3'	C4'	O5'	-
(IV)	C1'	C2'	C3'	C4'	O5'	-
(V)	C1'	C2'	C3'	C4'	O1'	-
	C1'	C2'	C3'	C5'	O2'	-
(VI), (VI')	C1'	C2'	C4'	C5'	O2'	-
(VII), (VII')	C1'	O'	C3'	C4'	O1'	C2'
	C1'	O'	C3'	C5'	O2'	C2'
(VIII), (VIII')	C1'	O'	C3'	C4'	O5'	C2'

*Branch going through O1'
**Branch going through C2'

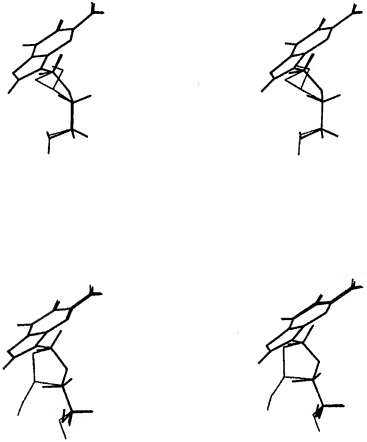


Fig.4. A stereoview of superposition of one low-energy conformation of compound II (thick line) with one of deoxyguanosine conformation (thin line).

could obtain the same level of superposition for inactive compounds (VII and VIII from Table 5) as well, but in this case the ΔU value would be overestimated (see e.g., the cases of D = 0.04 $\AA^2$ and ΔU = 8.0 kcal/mol for compound VII' or D = 0.05 $\AA^2$ and ΔU = 6.9 kcal/mol for compound VIII' in Table 5). Thus it can be concluded that simultaneous fulfillment of spatial similarity and low energy requirements allowed us to distinguish between active and inactive acyclic analogues of deoxyguanosine.

A similar problem was discussed in [15], however, the desired level of distinction between the analogues was not achieved. It

can be guessed that a possible reason for this failure was the description of spatial similarity of conformations in terms of torsional angles instead of the Cartesian coordinates of atoms. In our case the level of excellent spatial superposition is demonstrated in Fig.4, where deoxyguanosine and acyclovir molecules are shown.

CONCLUSIONS

1. The conformational mobility of acyclic analogues possessing and lacking antiviral activity is substantially different.

2. The spatial location of the oxygen atom, involved in enzymatic phosphorylation with respect to the base is not the sole factor determining the biological activity.

3. A computational algorithm which allows to distinguish between active and inactive analogues on the basis of energetical and spatial superposition levels for the conformations of a nucleoside and its acyclic analogue was elaborated.

4. The acyclic parts of biologically active molecules can be superposed on deoxyribose atoms by going through one of the two branches leading through either nonhydrogen substituents at C1' in the five-membered ring with accuracy of $D \leq 0.1$ Å^2 within the energy gap of 6 kcal/mol.

ACKNOWLEDGEMENT

Authors are grateful to Dr. T.Weselowski for his kind permission to read the manuscript of [15] before publication.

References

1. Chu, C.K. and Culter, S.J. (1986), J.Heterocyclic.Chem. 23, 289-319.
2. Phadtare, S., Zemlicka, J. (1987), Nucleic Acids Res., Symp. Ser. N 18, 25-28.
3. Lugovskoi, A.A., Dashevski, V.G., Kitaigorodski, A.J. (1972), Molek.Biol. 6, 449-456.
4. Lugovskoi, A.A., Dashevski, V.G., Kitaigorodski, A.J. (1972), Ibid. 6, 614-620.
5. Lugovskoi, A.A., Dashevski, V.G. (1972), Ibid. 6, 440-448.
6. Zhurkin, V.B., Poltev, V.I., Florentiev, V.L. (1980), Ibid. 14, 1116-1130.
7. Birnbaum, G.I., Cygler, M. and Shugar, D. (1984), Can.J.Chem., 62, 2646-2652.
8. Arnott, S., Dover, S.D. and Wonacott, A.J. (1969), Acta Cryst. B25, 2192-2206.

9. Momany, F.A., McGuire, R.F., Burgess, A.W. and Scheraga, H.A. (1975), J.Phys.Chem. 79, 2361-2381.
10. Sobol, I.M., Statnikov, R.B. (1981) In: Selection of Optimal Multicriterion Parameters. Moscow, pp.110 (in Russian).
11. Nyburg, S.C. (1974), Acta Cryst. B30, part I, 251-253.
12. Nikiforovich, G.V., Shenderovich, M.D., Balodis, J. (1981), Bioorg. Khim. 7, 179-188.
13. Datema, R., Johansson, N.G., Öberg, B. (1986), Chem.Scripta, 26, No 1, 49-53.
14. Keller, P.M., Fyfe, J.A.,Beauchamp, L., Lubbers, C.M., Furman, P.A., Shaeffer, H.J., Elion, G.B. (1981), Biochem. Pharm. 30, 3071.
15. Weselowski, T.A., Godzik, A. and Geller, M., J.Mol.Struct. THEOCHEM, 190 (1988), 105-112.
16. Weselowski, T.A., Godzik, A. (1987) Acta Biochim.Polonica, 34, 111-122.

radiation chemical yield measured in mol J^{-1}).

γ-Radiolysis. Quinone solutions were first saturated with N_2O gas in syringes as above, before being irradiated by a ^{60}Co source. Dose rates of 36 Gy min^{-1} were used, as measured by Fricke dosimetry.[30] Ion-selective electrodes for bromide (Russell pH Ltd.) and chloride (E.L.L.) were also used to monitor the release of leaving groups from quinones 1 and 2. Upon completion of irradiation, 20-mL aliquots of quinone solution were mixed with an ionic strength adjuster (20 mL of 0.2 M KNO_3 in the case of bromide ion detection, and 2 mL of 0.5 M ammonium acetate with 0.5 M acetic acid for chloride ions), and electrode measurements were carried out with continuous N_2 gassing to minimize re-oxygenation. Results were compared with predetermined calibration measurements.

Acknowledgment. This work was supported by the Cancer Research Campaign and by Grant CH-211 from the American Cancer Society. We thank Dr. G. M. Cohen (School of Pharmacy, University of London) for helpful discussions and for providing a sample of thiodione.

(30) Fielden, E. M. In *The Study of Fast Processes and Transient Species by Electron Pulse Radiolysis*; Baxendale, J. H.; Buse, F., Eds.; Reidel: Dordrecht, 1982; p 49.

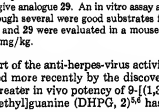
Synthesis and Anti-Herpes-Virus Activity of Acyclic 2′-Deoxyguanosine Analogues Related to 9-[(1,3-Dihydroxy-2-propoxy)methyl]guanine[1,2]

John C. Martin,*[3] Danny P. C. McGee,*[3] Gary A. Jeffrey, Doug W. Hobbs, Donald F. Smee, Thomas R. Matthews, and Julien P. H. Verheyden

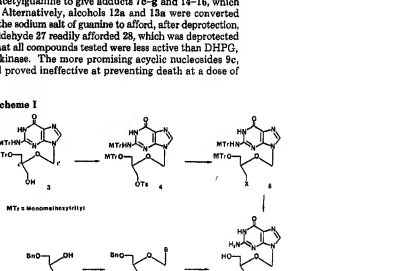
Syntex Research, Palo Alto, California 94304. *Received May 1, 1985*

Several "sugar" modified acyclic nucleoside analogues related to 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (DHPG, **2**) were synthesized and evaluated for antiviral activity. The preparation generally involved the condensation of the acetoxymethyl ether of alcohols **6c–g** and **10–12a** with diacetylguanine to give adducts **7c–g** and **14–16**, which were then deprotected to afford analogues **9c–g** and **17–19**. Alternatively, alcohols 12a and 13a were converted to iodides via their toxylates **12b** and **13b** and then reacted with the sodium salt of guanine to afford, after deprotection, analogues **22** and **23**. A crossed aldol–Cannizzaro reaction on aldehyde **27** readily afforded **28**, which was deprotected to give analogue **29**. An in vitro assay against HSV-1 showed that all compounds tested were less active than DHPG, though several were good substrates for the viral thymidine kinase. The more promising acyclic nucleosides **9c**, **19**, and **29** were evaluated in a mouse encephalitis model and proved ineffective at preventing death at a dose of 20 mg/kg.

The report of the anti-herpes-virus activity of acyclovir (1)[4] followed more recently by the discovery of the substantially greater in vivo potency of 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (DHPG, 2)[5,6] has stimulated a substantial research effort in the synthesis of acyclic guanosine analogues.[7]



Scheme I



Both acyclovir and DHPG are unusually selective as compared to other nucleoside antiviral agents.[8] The se-

(1) Contribution 206 from the Institute of Bio-Organic Chemistry, Syntex Research.

(2) Presented in part at the 185th National Meeting of the American Chemical Society, Seattle, WA; March 24, 1983; CARB 45.

(3) Current address: Pharmaceutical Research and Development, Bristol-Myers Co., Wallingford, CT 06492-7660.

(4) Schaeffer, H. J.; Beauchamp, L.; de Miranda, P.; Elion, G.; Bauer, D. J.; Collins, P. *Nature (London)* **1978**, *272*, 583.

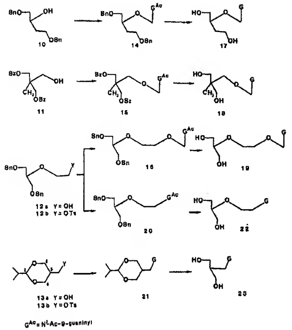
(5) (a) Martin, J. C.; Dvorak, C. A.; Smee, D. F.; Matthews, T. R.; Verheyden, J. P. H. *J. Med. Chem.* **1983**, *26*, 759. (b) Verheyden, J. P. H.; Martin, J. C. U.S. Patent 4 355 032, Oct 19, 1982. (c) Ogilvie, K. K.; Cheriyan, U. O.; Radatus, B. K.; Smith, K. O.; Galloway, K. S.; Kennell, W. L. *Can. J. Chem.* **1982**, *60*, 3005. (d) Field, A. K.; Davies, M. E.; DeWitt, C.; Perry, H. C.; Liou, R.; Germershausen, J. I.; Karkas, J. D.; Ashton, W. T.; Johnston, D. B. R.; Tolman, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 4139. (e) Schaeffer, H. J. In *Nucleosides, Nucleotides, and their Biological Applications*; Rideout, J. L., Henry, D. W., Beacham, L. M., Eds.; Academic Press: New York, 1983; pp 1–17.

(6) The structural formulas of DHPG (2) and the related acyclic nucleoside analogues have been depicted in a "ribose-like" conformation only to draw attention to the similarity in structure between these compounds and 2′-deoxynucleosides. In accordance with this representation, the two terminal carbons of the glycerol are referred to as the 3′ and 5′ positions.

(7) (a) Ogilvie, K. K.; Nghe Nguyen-Ba; Gillen, M. F.; Radatus, B. K.; Cheriyan, U. O.; Hanna, H. R. *Can. J. Chem.* **1984**, *62*, 241. (b) Ogilvie, K. K.; Hamilton, R. G.; Gillen, M. F.; Radatus, B. K.; Smith, K. O.; Galloway, K. S. *Can. J. Chem.* **1984**, *62*, 16. (c) Robins, M. J.; Hatfield, P. W. *Can. J. Chem.* **1982**, *60*, 547. (d) Parkin, A.; Harnden, M. R. *J. Heterocycl. Chem.* **1982**, *19*, 33. (e) Martin, J. C.; Jeffrey, G. A.; McGee, D. P. C.; Tippie, M. A.; Smee, D. F.; Matthews, T. R.; Verheyden, J. P. H. *J. Med. Chem.* **1985**, *28*, 358. (f) De Clercq, E.; Holy, A. *J. Med. Chem.* **1979**, *22*, 510. (g) Liu, M.-C.; Kuzmich, S.; Lin, T.-S. *Tetrahedron Lett.* **1984**, *25*, 613. (h) Robins, M. J.; Hatfield, P. W.; Balzarini, J.; De Clerq, E. *J. Med. Chem.* **1984**, *27*, 1486. (i) Kelley, J. L.; Kelsey, J. R.; Hall, W. R.; Krochmal, M. P.; Schaeffer, H. J. *J. Med. Chem.* **1981**, *24*, 753. (j) Zemlicka, J. *Nucleosides & Nucleotides* **1984**, *3*, 245.

0022-2623/86/1829-1384$01.50/0 © 1986 American Chemical Society

Scheme II



G^{Ac} = N^2-Ac-9-guaninyl
G = 9-guaninyl

lectivity of these analogues is due in part to the fact that they are appreciably phosphorylated only in virus-infected cells, where a virus-specified thymidine kinase of low substrate specificity converts the nucleoside analogues to monophosphates.[9] The monophosphates are next converted to diphosphates and then to the corresponding nucleoside triphosphates by cellular enzymes. The triphosphates prevent virus replication by inhibition of the viral DNA polymerase. Additional selectivity is realized at this stage because the host polymerase is less sensitive than the viral polymerase to the nucleoside triphosphate analogue.

We have been synthesizing a number of analogues of DHPG in order to further study the effects of structural modifications on antiviral activity and toxicity and now report the preparation and antiviral activity of a number of "sugar" modified DHPG analogues.

Chemistry. The synthesis of the DHPG analogues with one of the alcohol functionalities on the side chain replaced by another functionality is depicted in Scheme I. DHPG was converted to N^2,*O*-bis(monomethoxytrityl) derivative **3** in 43% yield. Tosylation of **3** gave **4**, which was converted to azide **5a** in 96% overall yield. Deprotection of **5a** with aqueous acetic acid gave azide **9a** (88%). Alternatively, reduction of **5a** with Raney nickel afforded **5b**, which was deprotected to furnish amine **9b** (78% overall).

Substituted glycerol derivatives **6c–g** were used for the synthesis of other analogues. Halohydrins **6c,d** were prepd. by the BF_3 etherate catalyzed opening of epifluorohydrin and epichlorohydrin with benzyl alcohol.[10] Ethers **6e,f** were synthesized by the treatment of **6d** with the appropriate sodium alcoxide. Propylene glycol derivative **6g** was prepared by the reaction of propylene oxide with sodium benzoxide. Alcohols **6c–g** were chloromethylated, treated with sodium acetate, and then reacted with N^2,9-diacetylguanine to give **7c–g** and **8c–g** (Table I). The isomers were separated by chromatography. The structures of the N^9-isomers **7** and **9** and the N^7-isomer **8** were con-

Table I. Physical Data

compd	% yield	mp, °C/solvent	formula
3	43	159–161/ethanol	$C_{49}H_{45}N_5O_5\cdot 0.5H_2O$
4	99	126–128/ethanol	$C_{56}H_{51}N_5O_8S$
5a	96	190–192/ethyl acetate–hexane	$C_{49}H_{44}N_8O_5\cdot H_2O$
5b	80	206–210 dec/methanol-dichloromethane	$C_{49}H_{46}N_6O_5\cdot 2H_2O$
6c	33	oil	$C_{10}H_{13}O_2F$
6e	75	oil	$C_{11}H_{16}O_3$
6f	64	oil	$C_{15}H_{22}O_3$
6g	46	oil	$C_{10}H_{14}O_2$
7c	18	167–170/methanol–ethyl acetate	$C_{18}H_{20}N_5O_4F$
7d	20	174–175/methanol–ethyl acetate	$C_{18}H_{20}N_5O_4Cl$
7e	16	140–141/ethyl acetate–hexane	$C_{19}H_{23}N_5O_5$
7f	19	146–148/ethyl acetate–hexane	$C_{23}H_{29}N_5O_5$
7g	15	173–175/ethanol	$C_{18}H_{21}N_5O_4$
8c	11	127–133/ethanol	$C_{18}H_{20}N_5O_4F$
8d	6	151–153/ethyl acetate–hexane	$C_{18}H_{20}N_5O_4Cl$
8e	3	94–95/ethyl acetate–hexane	$C_{19}H_{23}N_5O_5\cdot 0.5H_2O$
8f	5	102–103/ethyl acetate–hexane	$C_{23}H_{29}N_5O_5$
9a	88	207–208/water–methanol	$C_9H_{12}N_8O_3$
9b[a]	78	157–158/water–ethanol	$C_9H_{14}N_6O_3\cdot HCl\cdot 0.25H_2O$
9c	32	255–256/water–methanol	$C_9H_{12}N_5O_3F\cdot 0.5H_2O$
9d	50	206–208/water–methanol	$C_9H_{12}N_5O_3Cl$
9e	67	208–210/water–methanol	$C_{10}H_{15}N_5O_4\cdot 0.5H_2O$
9f	78	224–226/water–methanol	$C_{14}H_{21}N_5O_4$
9g[b]	91	275 dec/water/ethanol	$C_9H_{13}N_5O_3\cdot H_2O$
10	87	oil	$C_{18}H_{22}O_3$
11	50	oil	$C_{18}H_{20}N_5$
12a	50	oil	$C_{19}H_{24}O_4$
12b	92	oil	$C_{26}H_{30}O_6S$
13b	66	89–90/diethyl ether–petroleum ether	$C_{15}H_{22}O_5S$
14	11	122–124/ethyl acetate–hexane	$C_{26}H_{29}N_5O_8$
15	30	184–186/ethyl acetate–hexane	$C_{27}H_{27}N_5O_7$
16	25	133–135/ethyl acetate–ether	$C_{27}H_{31}N_5O_8$
17	55	222–223/water–methanol	$C_{10}H_{15}N_5O_4\cdot 0.5H_2O$
18	81	230–232/methanol	$C_{11}H_{17}N_5O_4\cdot H_2O$
19	67	167–169/ethanol	$C_{11}H_{17}N_5O_5$
20	4	144–146/ethyl acetate–hexane	$C_{26}H_{29}N_5O_5$
21	29	293–295/methanol	$C_{13}H_{19}N_5O_3$
22	50	220–222/methanol	$C_{10}H_{15}N_5O_4$
23	77	297–298/water–methanol	$C_9H_{13}N_5O_3$
24	98	243–244/methanol	$C_{10}H_{15}N_5O_4$
26	78	179–181/ethanol	$C_{23}H_{27}N_5O_4\cdot 0.5H_2O$
28	53	159–161/ethanol	$C_{30}H_{31}N_5O_5\cdot 1.5H_2O$
29[c]	85	>300/water	$C_{10}H_{15}N_5O_5$

[a] Mp reported 158–160 °C.[7g] [b] Previously disclosed without physical data.[8b] [c] Mp reported >300 °C.[15]

(8) Kelley, J. L.; Beauchamp, L. *Annu. Rep. Med. Chem.* **1983**, *18*, 139.
(9) (a) Smee, D. F.; Martin, J. C.; Verheyden, J. P. H.; Matthews, T. R. *Antimicrob. Agents Chemother.* **1983**, *23*, 676. (b) Keller, P. M.; Fyfe, J. A.; Beauchamp, L.; Lubbers, C. M.; Furman, P. A.; Schaeffer, H. J.; Elion, G. B. *Biochem. Pharmacol.* **1981**, *30*, 3071. (c) Cheng, Y-C.; Grill, S. P.; Dutschman, G. E.; Nakayama, K.; Bastow, K. F. *J. Biol. Chem.* **1983**, *258*, 12460. (d) Ashton, W. T.; Karkas, J. D.; Field, A. K.; Tolman, R. L. *Biochem. Biophys. Res. Commun.* **1982**, *108*, 1716. (e) St. Clair, M. H.; Miller, W. H.; Miller, R. L.; Lambe, C. U.; Furman, P. A. *Antimicrob. Agents Chemother.* **1984**, *25*, 191.
(10) Berecoechea, J.; Anatol, J. *Oleagineux* **1969**, *24*, 347; *Chem. Abstr. 71*, 90725*h*.

Scheme III

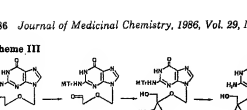


Table II. Phosphorylation of Nucleoside Analogues by Purified HSV-1 (F Strain) Thymidine Kinase and Antiviral Activities against HSV-1 (F Strain) in Cell Culture

compd	vel, % rel to thymidine	ID_{50}, μM	compd	vel, % rel to thymidine	ID_{50}, μM
1	23	0.5	9g	(28)[b]	(2.3)[b]
2	98	0.2 (0.5)[a]	17	111	95
9a	25	>100	18	100	>100
9b	<10	32 (100)[a]	19	23	7 (>100)[a]
9c	67	7 (>100)[a]	22	35	>100
9d	33	60	23		>100
9e	32	70	29	77	3.5[c] (55)[a]
9f	21	40			

[a] Antiviral activities against HSV-2 (G strain) in cell culture. [b] Biological data previously reported.[8b] [c] ID_{50} previously reported 54 μM.[15]

firmed by spectroscopic comparisons (^{13}C NMR,[11] UV) with the natural nucleosides. The N^9-isomers **7c–g** were deprotected by normal or transfer hydrogenation[12] and then ammonolysis to give **9c–g**.

Alcohols **10–13a** were used as starting materials for the synthesis of chain-length-modified analogues of DHPG (Scheme II). Alcohol **10** was synthesized by the treatment of 4-benzyloxy-1-butene oxide[13] with sodium benzoxide. Dibenzoate **11** was synthesized by the reaction of the corresponding triol with 2 equiv of benzoyl chloride. Hydroxyethyl derivative **12a** was prepared by reacting the sodium salt of 1,3-di-*O*-benzyl glycol with bromoacetaldehyde diethyl acetal followed by hydrolysis and reduction.

Alcohols **10–12a** were converted as described for the preparation of **7c** to their corresponding acetoxymethyl ethers and then condensed with diacetylguanine to give **14–18**. Standard deprotection afforded analogues **17–19**. Alternatively, alcohols **12a** and **13a**[14] were converted to their corresponding iodides via the tosylate intermediates **12b** and **13b** and then reacted with the sodium salt of guanine to give intermediates **20** (includes acetylation to aid purification) and **21**. Deprotection gave the free acyclic nucleosides **22** and **23**.

The trihydroxy analogue **29**, recently reported by Ogilvie and co-workers,[15] was synthesized by a method developed in this laboratory for the synthesis of 4′-hydroxymethylated nucleosides.[15] Acyclovir (**1**) was acetylated to give **24**, which in turn was monomethoxytritylated to **25** and then deacetylated to furnish **26**. Moffatt oxidation of **26** afforded intermediate aldehyde **27**, which was hydroxymethylated by treatment with paraformaldehyde and NaOH to give **28**. Deprotection of **28** with aqueous acetic acid afforded the desired analogue **29** (Scheme III).

Table III. Effects of Subcutaneous Treatment with **9c**, **19**, and **29** on an HSV-2 Encephalitis Infection in Mice

compd	dose, mg/kg	survivors/total	mean survival time, days
placebo		2/20 (10%)[a]	9.2 ± 1.9[b]
DHPG	20	10/16 (63%)[c]	13.5 ± 1.8[d]
9c	20	2/10 (20%)[c]	8.5 ± 1.1[c]
19	20	3/10 (30%)[c]	10.3 ± 1.5[d]
29	20	3/10 (30%)[c]	9.3 ± 0.75[c]

[a] Percent survival. [b] Standard deviation. [c] Statistical significance of $p < 0.05$ by two-tailed Fisher exact test. [d] Statistical significance of $p < 0.05$ by two-tailed Mann–Whitney test. [e] Not statistically significant.

Biological Results and Discussion

The antiviral activities of the "sugar" modified DHPG analogues were determined against herpes simplex virus type 1 (F strain) by plaque reduction in Vero cells. The ability of these analogues to function as substrates for a viral-specified thymidine kinase was also measured and compared with the above in vitro antiviral effect (Table II). The data in the table indicate that of the C-3′ (C-5′) substituted analogues, **9a–f**, the fluoro analogue **9c** is the more active member and is phosphorylated by the viral kinase at 67% the rate of thymidine. Although analogues **9a–f** are phosphorylated by the viral kinase at rates comparable to acyclovir, the attenuated antiviral activity exhibited by these compounds can possibly be attributed to the inefficient conversion of the substrate to the triphosphate or, once at that level, failure to inhibit the viral DNA polymerase. This is further evinced by the low antiviral activity of homologues **17** and **18** in spite of their phosphorylation by the viral kinase at a rate comparable to that of thymidine (100%). In addition, it has previously been demonstrated that certain analogues of DHPG[7a] and acyclovir[8b] were inactive as antivirals despite being substrates for the viral kinase.

Examination of the series of compounds **17–19**, **22**, **23**, and **29** clearly demonstrates that a change of one or two carbons or branching at various points on the DHPG "sugar" portion generally leads to decreased antiviral activity, although some affinity for the viral kinase is retained. Prominent among these are compounds **19** and **29**, which retained moderate in vitro antiviral activities with ID_{50} of 7 and 3.5 μM, respectively, against HSV-1.

It should be noted that compounds **9a–g** and **17**, due to the method of preparation, are in fact racemic mixtures. The two enantiomeric forms need not necessarily have the same antiviral activity.[17]

The in vivo efficacies of the more promising analogues **9c**, **19**, and **29** were compared with DHPG in a mouse encephalitis model (Table III). All three compounds tested showed no activity at a dose of 20 mg/kg in terms of survivor increase, but analogue **19** did statistically increase the mean survival time. In a related experiment in mice (HSV-2 encephalitis), compound **9b** showed a 31% increase in survivors at a dose of 500 mg/kg as compared to an equipotent dose of 200 mg/kg for acyclovir. Compound **9g** was inactive when tested at this high dose.

Experimental Section

Nuclear magnetic resonance spectra were recorded on samples dissolved in deuteriodimethyl sulfoxide unless otherwise stated.

(11) Strothers, J. B. *Carbon-13 NMR Spectroscopy*; Academic: New York, 1972; pp 469–473.
(12) (a) Jackson, A. E.; Johnstone, R. A. W. *Synthesis* **1976**, 685. (b) Anantharamaiah, G. M.; Sivanandaiah, K. M. *J. Chem. Soc., Perkin Trans. 1* **1977**, 490.
(13) Martin, J. C.; Smee, D. F.; Verheyden, J. P. H. *J. Org. Chem.* **1985**, *50*, 755.
(14) (a) Eliel, E. L.; Banks, H. D. *J. Am. Chem. Soc.* **1972**, *94*, 171. (b) Dekmezian, A. H.; Kaloustian, M. K. *Syn. Commun.* **1979**, *9*, 431.
(15) Ogilvie, K. K.; Nghe Nguyen-Ba; Hamilton, R. G. *Can. J. Chem.* **1984**, *62*, 1622.
(16) (a) Youssefyeh, R. D.; Verheyden, J. P. H.; Moffatt, J. G. *J. Org. Chem.* **1979**, *44*, 1301. (b) Jones, G. H.; Taniguchi, M.; Tegg, D.; Moffatt, J. G. *J. Org. Chem.* **1979**, *44*, 1309.
(17) Ashton, W. T.; Canning, L. F.; Reynolds, G. F.; Tolman, R. L.; Karkas, J. D.; Liou, R.; Davies, M.-E. D.; DeWitt, C. M.; Perry, H. C.; Field, A. K. *J. Med. Chem.* **1985**, *28*, 926.

For convenience and where applicable, the peak assignments of the two terminal carbons of the glycerol side chain are numbered 3′ and 5′. All chromatographic purifications were carried out on silica gel. Melting points were determined on a hot-stage microscope and are corrected.

N^3-(p-Anisyldiphenylmethyl)-9-[[1-(p-anisyldiphenylmethoxy)-3-hydroxy-2-propoxy]methyl]guanine (3). A mixture of 2 (8.18 g, 32 mmol), p-anisylchlorodiphenylmethane (21.7 g, 70 mmol), triethylamine (13.3 mL, 95 mmol), and 4-(dimethylamino)pyridine (0.06 g, 0.7 mmol) in DMF (100 mL) was magnetically stirred under a drying tube at 40 °C for 2 h; then methanol (10 mL) was added, and the solvents were evaporated. The residue was dissolved in ethyl acetate, washed with aqueous $NaHCO_3$ and water, dried over $MgSO_4$, and evaporated. The resulting oil was chromatographed (1:14 methanol/dichloromethane) and the product crystallized from ethanol to give 11.2 g (43%) of 3: UV λ_{max} (methanol) 279 nm (ε 13 000), 260 (12 000); ^1H NMR δ 10.63 (br s, 1 H, NH), 7.75 (s, 1 H, H-8), 7.66 (s, 1 H, NH), 7.24, 7.13, 7.05, 6.85, 6.70 (m, 28 H, aromatic), 5.04, 4.96 (AB, J = 11 Hz, 2 H, H-1), 4.39 (t, 1 H, OH), 3.77, 3.59 (s, 6 H, OCH_3), 3.47 (m, 1 H, H-4′), 3.05, 2.95 (ABM, 2 H, J = 1, 3, 11 Hz, CH_2OH), 2.77, 2.60 (ABM, 2 H, J = 5, 11 Hz, CH_2OMTr).

N^3-(p-Anisyldiphenylmethyl)-9-[[1-(p-anisyldiphenylmethoxy)-3-(p-toluenesulfonyloxy)-2-propoxy]methyl]guanine (4). A solution of 3 (11.5 g, 14.1 mmol) and p-toluenesulfonyl chloride (10.5 g, 55 mmol) in pyridine (100 mL) was kept at room temperature for 2 days, and then water (10 mL) was added. The solution was evaporated to dryness. The residue was dissolved in ethyl acetate, washed with water, dried over Na_2SO_4, and evaporated to give 11.6 g (100%) of 4 as a foam. An analytical sample was obtained by crystallization from ethanol: UV λ_{max} (methanol) 273 nm (ε 15 100), 260 (16 400); ^1H NMR δ 10.69 (br s, 1 H, NH), 7.76 (s, 1 H, H-8), 7.74 (br s, 1 H, NH), 7.66–6.67 (m, 32 H, aromatic), 5.03 and 4.84 (AB, J = 12 Hz, 2 H, H-1′), 3.77 (s, 3 H, OCH_3), 3.67 (s, 3 H, OCH_3), 3.60–3.30 (m, 3 H, H-4′, CH_2OTs), 2.63 (m, 2 H, CH_2OMTr), 2.45 (s, 3 H, CH_3).

N^2-(p-Anisyldiphenylmethyl)-9-[[1-(p-anisyldiphenylmethoxy)-3-azido-2-propoxy]methyl]guanine (5a). A solution of 4 (11.6 g, 14.1 mmol) and NaN_3 (5.0 g, 77 mmol) in DMF (100 mL) was heated at 105 °C for 3 days. The solution was diluted with ethyl acetate, washed with water, dried over Na_2SO_4, and evaporated to give 11.1 g (96%) of 5a as a foam. An analytical sample was obtained by crystallization from ethyl acetate/hexane: UV λ_{max} (methanol) 276 nm (ε 13 900), 260 (14 900); IR 2100 (N_3) cm^{-1}; ^1H NMR δ 10.68 (br s, 1 H, NH), 7.78 (s, 1 H, H-8), 7.68–7.35 (m, 28 H, aromatic), 5.03 and 4.95 (AB, J = 12 Hz, 2 H, H-1′), 3.77 (s, 3 H, OCH_3), 3.59 (s, 3 H, OCH_3), 3.54 (m, 1 H, H-4′), 3.06–2.55 (m, 4 H, H-3′, H-5′).

9-[[3-Amino-1-(p-anisyldiphenylmethoxy)-2-propoxy]-methyl]-N^2-(p-anisyldiphenylmethyl)guanine (5b). A mixture of 5a (11.1 g, 13.5 mmol) and Raney nickel[18] (60 g) in THF (150 mL) and ethanol (200 mL) was magnetically stirred under H_2 (1 atm) for 4 days. The mixture was filtered through Celite and the filtrate evaporated to dryness. The residue was chromatographed (1:9 methanol/dichloromethane) to give 5.63 g (80%) of 5b as a foam: UV λ_{max} (methanol) 276 nm (ε 14 700), 260 (16 400), 232 (30 300); ^1H NMR δ 7.79 (br s, 1 H, NH), 7.76 (s, 1 H, H-8), 7.37–6.68 (m, 28 H, aromatic), 5.03 and 4.80 (AB, J = 12 Hz, 2 H, H-1′), 3.77 (s, 3 H, OCH_3), 3.59 (s, 3 H, OCH_3), 3.40 (m, 1 H, H-4′), 2.82 (m, 2 H, CH_2OMTr), 2.40 (m, 2 H, CH_2NH_2).

9-[[1-Azido-3-hydroxy-2-propoxy]methyl]guanine (8a). A solution of 8a (1.24 g, 1.5 mmol) in 80% aqueous acetic acid (20 mL) was heated at 80 °C for 2 h and then evaporated to dryness. The residue was triturated with 1:3 ethyl acetate/hexane and then crystallized from water/methanol to give 0.37 g (68%) of 9a: UV λ_{max} (0.1 N HCl) an 275 nm (ε 9800), 256 (11 100), (0.1 N NaOH) 265 (10 400); IR 2110 (N_3) cm^{-1}; ^1H NMR δ 10.69 (br s, 1 H, NH), 7.83 (s, 1 H, H-8), 6.51 (br s, 2 H, NH_2), 5.47 (s, 2 H, H-1′), 4.87 (t, J = 5 Hz, 1 H, OH), 3.79 (m, 1 H, H-4′), 3.40 (m, 2 H, CH_2OH), 3.35, 3.27 (ABX, J = 6 and 12 Hz, 2 H, CH_2N_3); ^{13}C NMR (75.453 MHz) δ 156.77 (C-6), 153.76 (C-2), 151.30 (C-4), 137.53 (C-8), 116.38 (C-5), 78.04 (C-4′), 71.12 (C-1′), 60.59 (CH_2OH), 50.98 (CH_2N_3).

9-[(1-Amino-3-hydroxy-2-propoxy)methyl]guanine Hydrochloride (9b). A solution of 5b (8.63 g, 10.3 mmol) in 0.1 N methanolic HCl (500 mL) was kept at room temperature for 15 h and then evaporated to dryness. The residue was crystallized from water/ethanol to give 2.34 g (78%) of 9b: UV λ_{max} (0.1 N HCl) sh 276 nm (ε 8300), 267 (12 000), (0.1 N NaOH) 266 (10 800); ^1H NMR δ 10.85 (br s, 1 H, NH), 8.00 (br s, 3 H, NH_3Cl), 7.87 (s, 1 H, H-8), 6.69 (br s, 2 H, NH_2), 5.50 and 5.43 (AB, J = 11 Hz, 2 H, H-1′), 3.97 (m, 1 H, H-4′), 3.52 and 3.42 (after D_2O added, ABX, J = 5 and 11 Hz, 2 H, CH_2OH), 3.05 and 2.90 (after D_2O added, ABX, J_{AB} = 12 Hz, J_{AX} = 8 Hz, J_{BX} = 3 Hz, 2 H, CH_2NH_2).

1-Benzoyloxy-3-fluoro-2-propanol (6c). A solution of epifluorohydrin (10 g, 0.13 mol), benzyl alcohol (35 mL, 0.34 mol), and BF_3 etherate (1.5 mL, 15 mmol) was kept at 0 °C for 2 h and then at room temperature for 17 h. The solution was diluted with ethyl acetate, washed with saturated aqueous $NaHCO_3$, dried over Na_2SO_4, and evaporated to an oil. The residual oil was chromatographed with 3:10 ethyl acetate/hexane to give 7.94 g (33%) of 6c: ^1H NMR ($CDCl_3$, 100 MHz) δ 7.35 (s, 5 H, aromatic), 4.75, 4.20 (dd, J = 95, 3 Hz, 2 H, H-3), 4.57 (s, 2 H, benzylic), 4.0 (m, 1 H, H-2), 3.58 (m, 2 H, H-1), 2.40 (br s, 1 H, OH); ^{13}C NMR ($CDCl_3$, 22.62 MHz) δ 137.74, 128.61, 127.86 (aromatic), 86.97 (d, J = 169 Hz, C-F), 73.67 (benzylic), 70.07 (d, J = 6.6 Hz, C-1), 69.43 (d, J = 22 Hz, C-2).

1-Benzyloxy-3-methoxy-2-propanol (6e). A solution of 6d (20 g, 100 mmol) in 0.2 N methanolic $NaOCH_3$ was stirred for 16 h at room temperature then refluxed for 2 h. The resulting suspension was left at room temperature 16 h and was then concentrated and the residue partitioned between ethyl acetate and 5% aqueous HCl. The organic phase was washed successively with $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated to an oil, which was distilled at 101–105 °C/1 torr to afford 14.7 g (75%) of 6e: ^1H NMR ($CDCl_3$, 300 MHz) δ 7.35–7.30 (m, 5 H, aromatic), 4.56 (s, 2 H, benzylic), 4.0 (m, 1 H, CHOH), 3.55 and 3.50 (ABX, 2 H, J_{AX} = 4.4 Hz, J_{BX} = 6.3 Hz, J_{AB} = 6.8 Hz, CH_2OR), 3.47 and 3.43 (ABX, 2 H, J_{AX} = J_{AB} = 4.4 Hz, J_{BX} = 6.2 Hz, CH_2OR), 3.38 (s, 3 H, OCH_3), 2.53 (d, 1 H, OH); ^{13}C NMR ($CDCl_3$, 75.453 MHz) δ 138.23, 128.52, 127.88 (aromatic), 73.98 (CH_2CH_2), 73.54 (benzylic), 71.50 (CH_2OBn), 69.54 (CHOH), 59.20 (OCH_3).

N^2-Acetyl-9-[(1-benzyloxy-3-fluoro-2-propoxy)methyl]guanine (7e). Hydrogen chloride gas (dried through concentrated H_2SO_4) was bubbled into a stirred mixture of 6e (6.0 g, 33 mmol) and paraformaldehyde (2.0 g, 67 mmol) in dichloroethane (100 mL) at 0 °C until all the solids dissolved (3 h). The resulting solution was stored at 0 °C for 16 h, dried over $MgSO_4$, and evaporated to a clear oil. A solution of the oil and sodium acetate (4.0 g, 49 mmol) in DMF (75 mL) was kept at room temperature for 16 h and then evaporated to dryness. The residual oil was dissolved in ethyl acetate, washed with water and brine, dried over Na_2SO_4, and evaporated to a clear oil. A mixture of the oil, diacetylguanine (13.0 g, 55 mmol), bis(p-nitrophenyl)phosphate (0.10 g, 0.3 mmol), and sulfolane (22 mL) was heated at 95 °C with stirring for 5 days. The mixture was diluted with methanol and filtered through Celite. The filtrate was evaporated to dryness and chromatographed (1:19 methanol/dichloromethane) to give after crystallization from ethyl acetate 2.30 g (18%) of 7c: UV λ_{max} (methenol) 279 nm (ε 11 500), 258 (16 000); ^1H NMR (300 MHz) δ 8.14 (s, 1 H, H-8), 7.35–7.18 (m, 5 H, aromatic), 5.59 and 5.58 (AB, J = 3 Hz, 2 H, H-1′), 4.62–4.30 (m, 4 H, CH_2F, benzylic), 4.08 (m, 1 H, H-4′), 3.61–3.35 (m, 2 H, CH_2O), 2.18 (s, 3 H, CH_3); ^{13}C NMR (22.62 MHz) δ 173.60 ($COCH_3$), 154.91 (C-6), 148.80 (C-4), 148.08 (C-2), 139.99 (C-8), 137.97, 128.18, 127.70, 127.21 (aromatic), 120.22 (C-5), 82.76 (d, J = 168.4 Hz, CH_2F), 76.16 (d, J = 18.4 Hz, C-4′), 72.24 (benzylic) 72.04 (C-1′), 68.19 (d, J = 8.1 Hz, CH_2OBn), 23.67 ($COCH_3$).

N^2-Acetyl-7-[(1-benzyloxy)-3-fluoro-2-propoxy)-methyl]guanine (8c). From the above chromatographic purification of 7c, 1.39 g (11%) of 8c was isolated after crystallization from ethyl acetate: UV λ_{max} (methanol) 264 nm (ε 13 800); ^1H NMR (300 MHz) δ 8.38 (s, 1 H, H-8), 7.31–7.23 (m, 5 H, aromatic), 5.78 (s, 2 H, H-1′), 4.60–4.10 (m, 5 H, CH_2F, H-4′, benzylic), 3.45 (m, 2 H, CH_2O), 2.18 (s, 3 H, $COCH_3$); ^{13}C NMR (75.453 MHz) δ 173.32 ($COCH_3$), 157.44 (C-4), 152.44 (C-6), 147.13 (C-2), 144.96 (C-8), 137.94, 128.09, 127.54, 127.28 (aromatic), 110.90 (C-5), 82.70

(18) Raney nickel was prepared as described by Fieser, L. F.; Fieser, M. *Reagents for Organic Synthesis*; Wiley: New York, 1967; Vol. 1, p 729.

(d, J = 168.5 Hz, CH_2F), 75.87 (d, J = 17.8 Hz, C-4'), 74.53 (C-1'), 72.18 (benzylic), 68.18 (d, J = 8.4 Hz, CH_2OBn), 23.63 ($COCH_3$).

9-[(3-Fluoro-1-hydroxy-2-propoxy)methyl]guanine (9c). A mixture of 7c (1.05 g, 2.7 mmol), 2 drops of concentrated HCl, and 10% Pd/C in methanol (200 mL) and water (50 mL) was treated on a Parr hydrogenator with H_2 (50 psi) for 4 days. The mixture was filtered and the filtrate evaporated to dryness. A solution of the residue in concentrated NH_4OH (5 mL) and methanol (25 mL) was kept at room temperature for 18 h and then evaporated to dryness. The residue was crystallized from water/methanol to give 0.22 g (32%) of 9c: UV λ_{max} (methanol) sh 273 nm (ε 9030), 253 (13 600); ^1H NMR (300 MHz) δ 7.83 (s, 1 H, H-8), 6.53 (br s, 2 H, NH_2), 5.45 (s, 2 H, H-1'), 4.88 (t, J = 5 Hz, 1 H, OH), 4.60–4.25 (m, 2 H, CH_2F), 3.91–3.76 (m, 1 H, H-4'), 3.40 (m, 2 H, CH_2OH); ^{13}C NMR (22.62 MHz) δ 156.79 (C-6), 153.77 (C-2), 151.36 (C-4), 137.68 (C-8), 116.38 (C-5), 82.68 (d, J = 167.7 Hz, CH_2F), 77.49 (d, J = 18.4 Hz, C-4'), 71.23 (C-1'), 59.15 (d, J = 8.1 Hz, CH_2OH).

1,3-Di-*O*-benzyl-2-*O*-(2-hydroxyethyl)glycerol (12a). A mixture of NaH (2.0 g, 50%, 42 mmol, prewashed with hexanes) and 1,3-di-*O*-benzylglycerol[10] (10.0 g, 37 mmol) in DMF (25 mL) was heated at 50 °C for 0.5 h then cooled to 0 °C. Bromoacetaldehyde diethyl acetal (6.3 mL, 42 mmol) was added and the resulting solution heated at 50 °C for 17 h then evaporated. The residue was dissolved in ether, washed with water and brine, dried over Na_2SO_4, and evaporated. The resulting clear oil was chromatographed (2:8 ethyl acetate/hexane) to give 8.5 g (62%) of 1,3-di-*O*-benzyl-2-*O*-(2,2-diethoxyethyl)glycerol as a clear oil: ^1H NMR ($CDCl_3$) δ 7.30 (s, 10 H, phenyl), 4.64 (t, J = 5 Hz, 1 H, acetal-H), 4.54 (s, 4 H, benzylic), 3.77 (m, 1 H, CHO), 3.71–3.51 (m, 10 H, CH_2O), 1.18 (t, J = 7 Hz, 6 H, CH_3).

To the above acetal (25 g, 64.3 mmol) in THF (60 mL) and H_2O (6 mL) was added *p*-toluenesulfonic acid (300 mg), and the solution was refluxed 7 h then evaporated. The residual oil was partitioned between toluene and dilute $KHCO_3$, and the organic phase was dried with Na_2SO_4 and evaporated to a yellow oil. The crude aldehyde was redissolved in MeOH (60 mL) at 0 °C to which was added $NaBH_4$ (2.0 g, 52.8 mmol). The reaction was stirred for 30 min then evaporated. The residue was partitioned between toluene and dilute HCl (1×), dilute $KHCO_3$ (1×), and H_2O (1×); the organic phase was dried with Na_2SO_4 and evaporated to a golden oil. Fractional distillation afforded 9.33 g (48%) of pure 12a: bp 195–200 °C/0.5 torr; ^1H NMR ($CDCl_3$) δ 7.4 (s, 10 H, phenyl), 4.60 (s, 4 H, benzylic), 3.78 (m, 5 H, H-4' and ethoxy), 3.55 (d, 4 H, H-3', H-5'), 3.05 (br, 1 H, OH).

5-[(*p*-Toluenesulfonyloxy)methyl]-2-isopropyl-1,3-dioxane (13b). A solution of alcohol 13a (1 g, 6.17 mmol)[14] and *p*-toluenesulfonyl chloride (1.43 g, 7.5 mmol) in pyridine (10 mL) was stirred for 16 h at room temperature and then evaporated. The residue was redissolved in dichloromethane and partitioned with aqueous Na_2CO_3, dried ($MgSO_4$), and evaporated to a yellow oil. Chromatography (CH_2Cl_2) followed by crystallization from diethyl ether–petroleum ether yielded 1.3 g (66%) of 13b: ^1H NMR δ 7.78 (d, 2 H, J = 8.4 Hz, aromatic), 7.37 (d, 2 H, J = 8.6 Hz, aromatic), 4.10 (d, 1 H, J = 5.2 Hz, H-2), 4.05, 3.45 (ABX, 4 H, J_{AX} = 5 Hz, J_{BX} = 11.2 Hz, J_{AB} = 11.4 Hz, H-4, H-6), 3.80 (d, 2 H, J = 5.7 Hz, CH_2OTs), 2.46 (s, 3 H, aromatic CH_3), 2.32 (m, 1 H, H-5), 1.77 (m, 1 H, CH), 0.90 (d, 6 H, J = 6 Hz, CH_3).

9-[(2-Isopropyl-1,3-dioxan-5-yl)methyl]guanine (21). A suspension of the sodium salt of guanine (1.24 mmol), 13b (3.9 g, 1.24 mmol), and NaI (0.6 g, 4 mmol) in DMF (15 mL) was heated at 100–110 °C for 16 h then cooled, diluted with 20% methanol–dichloromethane (70 mL), filtered through Celite, and evaporated. The residue was chromatographed (15% methanol–CH_2Cl_2), and crystallization from methanol afforded 1.12 g (29%) of 21: UV λ_{max} (0.1 N HCl) 281 nm (ε 7700), 254 (10 900), (0.1 N NaOH) 288 (10 300), sh 254 (9450); ^1H NMR δ 7.63 (s, 1 H, H-8), 6.47 (br s, 2 H, NH_2), 4.16 (d, 2 H, H-2'), 4.87, 3.43 (ABX, 4 H, J = 4.7, 11 Hz, H-4', H-6'), 3.75 (d, 2 H, J = 7.3 Hz, CH_2-G), 2.40 (m, 1 H, H-5'), 1.69 (m, 1 H, CH), 0.83 (d, 6 H, J = 6 Hz, CH_3).

9-[3-Hydroxy-2-(hydroxymethyl)prop-1-yl]guanine (23). A solution of 21 (254 mg, 0.87 mmol) in 90% aqueous trifluoroacetic acid was stirred at room temperature for 20 min and then evaporated. The residue was coevaporated 3 times with ethanol, triturated twice with acetone, and the resulting solid crystallized from methanol/water to afford 23 (159 mg, 77%) in two crops: UV λ_{max} (0.1 N HCl) 273 nm (ε 7300), 253 (11 000), (0.1 N NaOH) 288 (9990), 254 (9160); ^1H NMR δ 7.89 (s, 1 H, H-8), 6.46 (br s, 2 H, NH_2), 4.6 (t, 2 H, OH's), 3.96 (d, 2 H, J = 6.8 Hz, CH_2), 3.94 (m, 4 H, CH_2OH), 2.02 (m, 1 H, CH).

9-[(2-Acetoxyethoxy)methyl]guanine (24). A mixture of 1 (3.0 g, 13.3 mmol) and 4-(dimethylamino)pyridine (0.30 g, 2.5 mmol) in acetic anhydride (100 mL) was vigorously stirred at room temperature for 5 days and then evaporated to dryness. The residue was crystallized from methanol to give 3.46 g (98%) of 24: UV λ_{max} (methanol) sh 275 nm (ε 10 000), 254 (14 000); ^1H NMR δ 10.69 (br s, 1 H, NH), 7.83 (s, 1 H, H-8), 6.53 (br s, 2 H, NH_2), 5.37 (s, 2 H, NCH_2O), 4.08 (t, J = 5 Hz, 2 H, CH_2OAc), 3.68 (t, J = 5 Hz, 2 H, CH_2O), 1.97 (s, 3 H, $COCH_3$).

N^2-(*p*-Anisyldiphenylmethyl)-9-[(2-hydroxyethoxy)-methyl]guanine (26). A solution of 24 (2.96 g, 11.1 mmol), *p*-anisylchlorodiphenylmethane (4.35 g, 15.6 mmol), triethylamine (4.0 mL, 29 mmol), and 4-(dimethylamino)pyridine (0.30 g, 2.5 mmol) in DMF (50 mL) was heated at 50 °C for 23 h, and then methanol (5 mL) was added. The solution was evaporated to dryness. The residue was dissolved in dichloromethane, washed with saturated aqueous $NaHCO_3$, dried over Na_2SO_4, and evaporated to dryness. The residue was chromatographed (1:15 methanol/dichloromethane) to give 5.09 g (85%) of 25 as a foam. A solution of 25 (3.97 g, 7.35 mmol) and concentrated NH_4OH (10 mL) in methanol (50 mL) was kept at room temperature for 3 days and then evaporated to dryness. The residue was crystallized from ethanol to give 2.85 g (78%) of 26: UV λ_{max} (methanol) 277 nm (ε 14 000), 260 (15 400); ^1H NMR δ 10.65 (br s, 1 H, NH), 7.69 (br s, 1 H, NH), 7.68 (s, 1 H, H-8), 7.17–7.35 (m, 12 H, aromatic), 6.86 (d, J = 9 Hz, 2 H, aromatic), 4.87 (s, 2 H, NCH_2O), 4.51 (t, J = 5 Hz, 1 H, OH), 3.72 (s, 3 H, OCH_3), 3.21 (m, 2 H, CH_2OH), 2.93 (t, J = 5 Hz, 2 H, CH_2O).

N^2-(*p*-Anisyldiphenylmethyl)-9-[[1,3-dihydroxy-2-(hydroxymethyl)-2-propoxy]methyl]guanine (28). A solution of 26 (1.63 g, 3.28 mmol), dicyclohexylcarbodiimide (2.60 g, 12.6 mmol), and methylphosphonic acid (0.20 g, 2.3 mmol) in dry Me$_2$SO (150 mL) was magnetically stirred at 15 °C for 4 h and then at room temperature for 18 h. The resulting suspension was cooled to 18 °C, and a solution of oxalic acid dihydrate (90 mg) in methanol (9 mL) was added. The suspension was filtered and the filtrate evaporated to dryness on a Kugelrohr apparatus (60 °C/1 torr). The residue was chromatographed (1:12 methanol/dichloromethane) to give 1.32 g (82%) of 27 as a white foam. A solution of 27 (1.07 g, 2.17 mmol), 37% aqueous formaldehyde (7.8 mL), and 1 N sodium hydroxide (7.8 mL) in THF (50 mL) and water (50 mL) was kept at room temperature for 18 h. Ammonium chloride (1.5 g) was then added and the solution evaporated to approximately 40 mL. The resulting solution plus the precipitated gum was partially dissolved in a mixture of water (200 mL) and dichloromethane (300 mL). The dichloromethane phase was combined with the residual gum and the mixture evaporated to dryness. The residue, preabsorbed onto silica gel (25 g), was chromatographed (1:6 methanol/dichloromethane) to give 0.64 g (53%) of 28 as a white solid. An analytical sample was crystallized from ethanol: UV λ_{max} (methanol) 277 nm (ε 13 150), 260 (14 800); ^1H NMR δ 10.67 (br s, 1 H, NH), 7.70 (br s, 1 H, NH), 7.65 (s, 1 H, H-8), 7.15–7.35 (m, 12 H, aromatic), 6.86 (d, J = 9 Hz, 2 H, aromatic), 5.01 (s, 2 H, NCH_2O), 4.35 (t, J = 5 Hz, 3 H, OH), 3.72 (s, 3 H, OCH_3), 3.25 (d, J = 5 Hz, 6 H, CH_2OH).

9-[[1,3-Dihydroxy-2-(hydroxymethyl)-2-propoxy]-methyl]guanine (29). A solution of 28 (272 mg, 0.49 mmol) in 80% aqueous acetic acid was heated at 80 °C for 2 h and then evaporated to dryness. The residue was triturated with ethyl acetate and then recrystallized from water to give 119 mg (85%) of 29: UV λ_{max} (methanol) sh 273 nm (ε 9300), 253 (13 300), (0.1 N HCl) sh 276 (8500), 256 (12 100), (0.1 N NaOH) 266 (11 200), 256 (11 100); ^1H NMR δ 10.62 (br s, 1 H, NH), 7.77 (s, 1 H, H-8), 6.46 (br s, 2 H, NH_2), 5.53 (s, 2 H, NCH_2O), 4.66 (t, J = 5 Hz, 3 H, OH), 3.51 (d, J = 5 Hz, 6 H, CH_2OH).

Plaque Assays. Experiments were conducted with Vero cells infected with HSV-1 (F strain) and then treated with the nucleoside analogue as described previously.[26] Fifty percent inhibitory doses (ID_{50}) are defined as doses causing a 50% reduction in plaque numbers compared to untreated controls.

Thymidine Kinase Assay. The assay was performed by the methods of Doberson and Greer,[19] using HSV-1 (strain F)-affinity-purified kinase.[20] Reaction mixtures contained 100 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM NaF, 1 mg BSA/mL, 0.5 mM [γ-^{32}P]ATP (20 μCi/μM), 0.2 mM nucleoside analogue, and enzyme in a 0.1 mL volume. The [γ-^{32}P]ATP required for the assay was from ICN Chemical and Radioisotope Div., Irvine, CA; thymidine came from Sigma Chemical Co., St. Louis, MO. The thymidine kinase affinity gel required for enzyme purification was prepared by the Cheng procedure,[21] and the results from the deoxynucleoside kinase assays were expressed as relative phosphorylation rates, a method that has been used by others.[20,22]

Animal Studies. Swiss-Webster female mice (Simonsen Laboratories, Gilroy, CA), weighing approximately 20 g each, were infected intraperitoneally with 5×10^4 PFU of HSV-2 (strain G). This challenge was approximately equivalent to 10 50% lethal doses. DHPG and the nucleoside analogues were administered subcutaneously once a day for 4 days starting 24 h postinfection. Deaths were recorded for 21 days after infectin.

Acknowledgment. The assistance of Dr. M. Maddox, J. Nelson, and L. Kurz in obtaining and interpreting NMR spectra is gratefully acknowledged. Technical support for the animal studies was provided by V. R. Freitas and B. P. Binko.

(19) Doberson, M. J.; Greer, S. *Anal. Biochem.* **1975**, *67*, 602.
(20) Fyfe, J. A.; Keller, P. M.; Furman, P. A.; Miller, R. L.; Elion, G. B. *J. Biol. Chem.* **1978**, *253*, 8721.
(21) Cheng, Y.-C. *Methods Virol.* **1978**, *51*, 365.
(22) Cheng, Y.-C.; Dutschman, G.; Fox, J. J.; Watanabe, K. A.; Machida, H. *Antimicrob. Agents Chemother.* **1981**, *20*, 420.

Synthesis and Antiviral Activity of Certain Nucleoside 5′-Phosphonoformate Derivatives

Morteza M. Vaghefi,*[†] Patricia A. McKernan,[†] and Roland K. Robins[†]

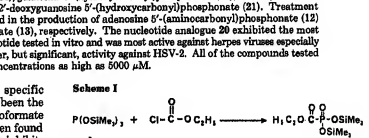
Department of Chemistry, Cancer Research Center, Brigham Young University, Provo, Utah 84602, and Nucleic Acid Research Institute, Costa Mesa, California 92626. *Received December 20, 1985*

(Ethoxycarbonyl)phosphonic dichloride (3) was synthesized by chlorination of bis(trimethylsilyl) (ethoxycarbonyl)phosphonate with thionyl chloride. Adenosine 5′-(ethoxycarbonyl)phosphonate (4), guanosine 5′-(ethoxycarbonyl)phosphonate (5), 2′-deoxyadenosine 5′-(ethoxycarbonyl)phosphonate (18) and 2′-deoxyguanosine 5′-(ethoxycarbonyl)phosphonate (19) were synthesized by coupling of compound 3 with adenosine, guanosine, 2′-deoxyadenosine, and 2′-deoxyguanosine, respectively. Alkaline treatment of 4, 5, 18, and 19 gave the corresponding adenosine 5′-(hydroxycarbonyl)phosphonate (14), guanosine 5′-(hydroxycarbonyl)phosphonate (15), 2′-deoxyadenosine 5′-(hydroxycarbonyl)phosphonate (20), and 2′-deoxyguanosine 5′-(hydroxycarbonyl)phosphonate (21). Treatment of 4 and 5 with methanolic ammonia resulted in the production of adenosine 5′-(aminocarbonyl)phosphonate (12) and guanosine 5′-(aminocarbonyl)phosphonate (13), respectively. The nucleotide analogue 20 exhibited the most potent antiviral activity of this group of nucleotide tested in vitro and was most active against herpes viruses especially HSV-2. The nucleotide analogue 21 had lower, but significant, activity against HSV-2. All of the compounds tested were nontoxic to confluent Vero cells at concentrations as high as 5000 μM.

Compounds which selectively inhibit virus specific functions but have little effect on host cells have been the target of many recent investigations.[1,2] Phosphonoformate (Foscarnet), an analogue of pyrophosphate, has been found to be a potent antiviral agent.[3] Phosphonoformate inhibits viral reverse transcriptase[4] and DNA polymerase[5] and is active against herpes simplex virus (HSV-1)[6,7] and certain other viruses.[8-10] Recently, elimination of replication of human T-cell lymphotropic virus type III (HTLV-III) with phosphonoformate at a nontoxic dose has been reported.[11,12] This study showed that the activity of reverse transcriptase of HTLV-III has been reduced drastically by phosphonoformate. Beldekas and co-workers studied the effect of foscarnet on the nature and frequency of T-cell differentiation defect seen in AIDS and in certain people with LAS.[13] These investigators reported that foscarnet permits T-cell colony growth and expansion in the cell culture.

Phosphonoformate has been used topically for treatment of HSV-1 infection in laboratory animals with satisfactory results.[14] Phosphonoformate has also been used in a 3% cream formulation, which is not irritating to the skin, and in clinical testing shortens the time mucocutaneous HSV-1 and HSV-2 lesions require to reach the stage of crusting and reduces the appearance of new vesicles.[15] Topical treatment of recurrent genital herpes with foscarnet cream has also been claimed to accelerate healing in man.[16]

Scheme I



It appears that phosphonoformate binds to the pyrophosphate "well" of the viral DNA and RNA polymerase.[4,5]

(1) Oberg, B. In *Problems in Antiviral Therapy*; Stuart-Harris, S. C. H., Oxford, J., Eds.; Academic Press: London 1983; p 35. Also, see: Lambert, H. P. *Ibid.* p 109.
(2) Alhadeen, H. S. et al. In *Antiviral Chemotherapy: Design of Inhibitors of Viral Function*; Gauri, K. K. Ed.; Academic Press, New York, 1981; p 89. Also, see: Helgstrand, E. et al. *Ibid.* p 139 and Erikson, B. et al. *Ibid.* p 161.
(3) Stenberg, K. *Biochem. Pharmacol.* **1981**, *30*, 1005.
(4) Erikson, B.; Steining, G.; Oberg, B. *Antiviral Res.* **1982**, *2*, 81.
(5) Erikson, B.; Oberg, B.; Wahren, B. *Biochim. Biophys. Acta* **1982**, *695*, 115.
(6) Helgetraad, E.; Erikson, B.; Johansson, N. G.; Lannero, B.; Larsson, A.; Misiorny, A.; Noren, J. O.; Sjoberg, B.; Stenberg, K.; Stening, G.; Stridh, S.; Oberg, B.; Alenius, S.; Philipson, L. *Science (Washington, DC)* **1978**, *201*, 819.
(7) Svennerholm, B.; Vahline, A.; Lycke, E. *Proc. Soc. Exp. Biol. Med.* **1979**, *61*, 115.

[†] Present address: Nucleic Acid Research Institute.

0022-2623/86/1829-1389$01.50/0 © 1986 American Chemical Society